Chapter 5: Discussion
Cells respond to a variety of different signals, most of which are perceived by
membrane-bound receptors at the cell surface. Some of these receptors can activate
heterotrimeric G proteins, which regulate a variety of enzymes activity and ion channels
in signal transduction. Despite the significant progress made in plant signal transduction,
the G protein signaling in plant remains elusive.

Heterotrimeric G proteins are composed of $\alpha$, $\beta$, and $\gamma$ subunits. There are 10 $\alpha$-subunits,
7 $\beta$-subunits, and 2 $\gamma$-subunits cloned from various plant species including Arabidopsis,
tomato, rice, maize and tobacco. Studies with recombinant proteins have revealed the
characteristics of plant $\alpha$ subunits, and some information is available on the interaction
among the subunits. Recently, it has been reported that loss-of-function in the $G\alpha$ and
$G\beta$ subunit in rice and Arabidopsis, and the result of antisense approach in rice, all led to
abnormal morphology in these plants (Ashikari et al., 1999; Fujisawa et al., 1999;
Ueguchi-Tanaka et al., 2000; Ullah et al., 2001, 2002; Wang et al., 2001). Though some
evidences suggest some role of G protein, but the detailed mode of their action still
remain unknown. To better understand the signaling role of G proteins in plants, we have
cloned, characterized, and functional analysis of G proteins from pea.

5.1 Cloning of G proteins from pea

5.1.1 Molecular cloning of cDNA of $G\alpha$ and $G\beta$ subunits

$G\alpha$ I and $G\alpha$ II subunits were cloned by RT-PCR approach. After complete nucleotide
sequencing of $G\alpha$ I and $G\alpha$ II, we found few differences between these and the
previously reported two G protein $\alpha$ subunits (PGA1 and PGA2). There are 12
nucleotide changes, which resulted in 8 amino acid changes in $PsG\alpha$ I (AF537218) as
compared to the PGA1 (U97043), while only 3 nucleotide changes, which resulted in 3
amino acids changes between $PsG\alpha$ II (AF533438) vs. PGA2 were observed (U97044).
The result suggests that the $PsG\alpha$ I and $PsG\alpha$ II could be the variants of the already
reported subunits. On comparison of $PsG\alpha$ I with $PsG\alpha$ II sequences, it showed that they
have 86% identity and 6% similarity, while the reported PGA1 and PGA2 share 88%
identity, and 5% similarity (Marsh and Kaufman, 1999).

Computational analysis of the sequence showed that the ORF of $PsG\alpha$ I encodes a
protein of 384 amino acid residues with a calculated molecular weight of 44.5 kDa and
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an estimated pI of 5.81. The ORF of PsGα II also encodes a protein consisting of 384 amino acid, with a calculated molecular of Gα II is 44.48 kDa and the estimated pI of 5.70. The deduced amino acid sequences of PsGα I and PsGα II subunits were found to contain all the functional domains that found in plant Gα subunit, such as a myristylation site (MGXXXS), four GTP-binding sites, a cholera toxin-binding site, three effector-binding sites and two receptor-binding sites (Fig. 8.4b), which have been identified in the mammalian α subunits.

In the bovine α subunit designated Gt, the first 22 amino acid residues at the N-terminus have been shown to participate in the interaction with the βγ subunit (Noel et al., 1993; Lambright et al., 1994). It has been proposed that coiled-coil structures are necessary for the interaction between the α subunit and the βγ subunit, and the N-terminal part of Gt has been suggested to exist as a coiled-coil segment (Lupas et al., 1992). The GTP-binding regions of mammalian α subunits have been identified by biochemical studies (Simon et al., 1991) and analyses of crystal structure (Noel et al., 1993; Lambright et al., 1994). Four GTP binding regions were found in PsGα I and PsGα II sequence. The GTP-binding regions are highly conserved among all plant Gα subunits including PsGα I and PsGα II subunits (Fig. 8.5). Two receptor-binding regions are located in the N-terminal and C-terminal part, majority variety was observed in N-terminal region in all plant Gα, PsGα I and PsGα II in particular, while C-terminal region sequence are more conserved.

Four positive Gβ clones were isolated after screening cDNA library. It was found that they possess same ORF with different UTR. The deduced amino acid sequence revealed a protein consisting of 377 amino acid residues with predicted molecular of about 41 kDa and pI of 7.04. Structural analysis revealed that four typical repetitive segments of Trp-Asp-40 amino acid repeats (WD-40 repeats) were present in PsGβ sequence, while seven WD-40 were reported in animal system and in tobacco β subunit (Ishida et al., 1993). Although this repeat unit is characterized by WD-40 repeats (Simon et al., 1991) and shows some degeneracy, the spacing of each repeat (i.e. 40 a.a.) is rather strict. This well-conserved motif was discovered in subtypes Gβ from human and later in a divergent group of organisms. In addition to G protein β subunits, a large number of proteins from yeast to human have been shown to contain the WD-40 motif. They have
very diverse functions, including cell cycle regulation, RNA splicing, regulation of Ras function, transcriptional repression, and actin binding. These proteins have different sizes (from 318 to >800 residues) and various numbers (five to eight) of WD-40 repeats. Most likely the WD-40 motif is involved in a general process, possibly playing a role in protein-protein recognition.

Gα and Gβ subunits have been reported from Arabidopsis, rice, tobacco, potato, oat (Fujisawa et al., 2001; Assmann, 2002). The deduced amino acid sequence of PsGα and PsGβ were compared with the reported sequences from other plants, and it showed significant homology. It suggests that the G protein have been conserved during evolution, thereby reflecting on the fundamental importance of G protein signaling in plant kingdom. However, in both Gα and Gβ subunits, variable regions can be seen in the N-terminals (Fig. 8.5 and 8.30), which are thought to serve as receptor binding and α/βγ subunit binding domain, may be leading the G proteins transduce signals from a variety of receptors to a variety of targets.

5.1.2 Protein expression and characterization

The ORF of Gα and Gβ subunits from pea were cloned and overexpressed in bacterial system. The purified protein was functionally characterized. It was found that the PsGα I contained GTPase activity as well as GTP binding activity, while PsGβ had no such activity. Similarly, Arabidopsis and rice Gα subunits, GPA1 and RGA1 have been shown to contain GTPase activity (Wise et al., 1997; Iwasaki et al., 1997).

The effect of other subunits of G proteins and PLC on the GTPase activity of Gα was also determined. It was observed that PsGβ has no effect on GTPase activity of PsGα but PLC-δ from pea could stimulate the activity significantly. This is a novel finding clearly suggesting that PLC-δ is one of the effector molecules of Gα subunit. Whether this interaction has any effect on the activity of PLC still needs to be investigated. It has been reported in plants that G protein activation stimulated PLD activity (Munnik et al., 1995). In animal system it has been shown that Gαq stimulates the PLC-β activity (Exton, 1997), and the βγ subunits modulate PLC-β activity (Singer et al., 1997).
In plant, only PLC-δ isoform has been cloned, and this is the first time that PLC stimulation Gα GTPase activity has been demonstrated. In animal system, it has been reported that PLC-β has the ability to activate intrinsic GTPase activity of Gαq 2000-fold. *In vitro* studies involving monoclonal antibodies raised against PLC-β blocked the stimulating activity of Gq as well as the Gq GAP (GTPase activity protein) activity of PLC-β proving that the source of GAP activity is PLC-β itself (Berstein et al., 1992). Studies suggest that the GAP activity of PLC-β lies in its carboxyl terminal region and is included in the region previously shown to be important for responsiveness to Gq (Paulssen et al., 1996).

### 5.1.3 Genomic organization of G proteins in pea

We have isolated two α subunits, PsGα I and PsGα II, which were slightly different than the published α subunits PGA1 and PGA2 (Marsh and Kaufman, 1999). Southern blot analysis of pea genomic DNA using PsGα I or PsGαII as probe showed multiple bands. However, it was found that PsGα I genome clone is 5 kb long, together with evidence of Southern and cDNA library screening showed by Marsh and Kaufman (1999), it seems that PsGα I is similar PGA1 and PsGαII is similar PGA2, the only two α subunits presented in pea genome. So far only in soybean it was reported to contain two α subunits (SGA1, Kim et al., 1995; SGA2, Gotor et al.1996), other plants only contain one copy of Gα subunit.

One Gβ subunit was isolated from pea. Southern blot analysis of pea genomic DNA using PsGβ cDNA as probe revealed that only one β subunit is present in the pea genome, suggesting that Gβ is a single copy gene. Early, two Gβ were cloned from wild oat (AfGβ1 and AfGβ2, Jones et al., 1998), while other plants contain only one Gβ subunit (Assmann, 2002).

In plant kingdom till to date the Gγ was isolated only from Arabidopsis (Mason and Botella, 2000, 2001). Our attempt to isolate Gγ from pea has failed so far. However, pea genomic DNA Southern blot hybridized with Arabidopsis Gγ DNA as probe, revealed multiple bands (Fig. 8.45), which suggested that more than one Gγ is present in pea.
Genome. It is reasonable that Arabidopsis, whose genome is much smaller than pea, has two γ subunits.

Mammals possess ~20 Gα, ~5 Gβ and at least 12 Gγ subunits (Seack et al., 1998, Cook et al., 2001), together with more than 1000 GPCRs. There are thus large number of different G proteins (20α x 5Gβ x 12Gγ combination) attached to different GPCRs to mediate in G protein signaling. Interesting, each plant genome contains only one or two genes for each subunit. Yet plant heterotrimeric G proteins have been proposed to mediate different signals, such as plant hormones (Hooley, 1999; Lovegrove and Hooley 2000), elicitor (Blumward et al., 1998) and light (Barbier-Brygoo et al., 1997; barnes et al., 1997). An interesting question is how a single or a couple of species of G proteins carry out these different function selectively. Further there is no homology of GPCRs found in Arabidopsis genome, and only one putative GCR1 was reported (Colucci et al., 2002). It is possible that there may be several species of the α or β subunits in some plant species, but may not have been detected because of much lower level of sequence homology. As far as one knows, there is no other sequence highly homologous to the GPA1 in the Arabidopsis genome sequence (Fujisawa, 2001). As far as pea is concerned, our data suggested that there are two Gα, one β and at least two Gγ in pea genome.

Genomic clones of Gα and Gβ were isolated from pea genome. PsGα II cDNA size is 1151 bp, while its genomic clone is 3351 bp. It has 12 introns and 13 exons. PsGβ cDNA is 1131 bp, but the genomic DNA is 4087 bp. It has 5 introns and 6 exons. On comparison of genomic organization of PsGα II and PsGβ with the Arabidopsis Gα and Gβ subunits, it was observed that both Gα II and Gβ have not only the same number of introns and exons, but also the same size of exon. The only difference is in the size of introns. The sizes of the introns in G protein range from 87 to 439 bp (Gα), from 89 to 1326 bp (Gβ), while the size of the exons varies between 38-175 bp (Gα), and 33-425 bp (Gβ). Plant introns are generally shorter than those in vertebrates and are usually <150 nucleotides in length; size between 60 to 10,000 nucleotides have been reported for plant introns (Lorkovic et al., 2000). Here, Gβ clone contained two long introns: 938 bp and 1326 bp.
5.1.4 Isolation of G protein promoter

The amplified fragment for the promoter region was 497 bp in length and showed the presence of a 35 bp overlap with the 5’UTR of Gα I (U97043). For Gβ gene promoter, the fragment was 379 bp in length and showed the presence of a 39 bp overlap with the 5’UTR of PsGβ (AF145976).

A search for putative cis-acting elements in the promoter regions for PsGα I and PsGβ revealed that both of them contain Dof binding site (AAAG), CAAT box, GATA box, E box, pollen and root specific motifs, light regulatory elements, GT-1 site etc. While AMY box, CATATG motif, GTGA and I box were only present in promoter of Gα I, and Inr motif, LTRE sequence, RAV1 binding sequence (CAACA) and WRKY binding site (TTGAC) were found only in Gβ promoter (Fig. 8.1 and 8.2.) Interestingly, both Gα and Gβ promoter did not have a canonical TATA box in the sequence. Thus the Gα and Gβ promoter is probably TATA-less. Especially Gβ promoter contains 4 Inr motifs, which are only present in TATA less promoter that responds to light (Nakamura et al., 2002).

Pollenspecific regulatory elements have the consensus AGAAA and have been described in the pollen specific genes lat52 (Tomato), Zm13 (Maize), Ps1 (Rice) and Npgl (Tobacco) (Bate and Twell, 1998). In the Gα and Gβ promoter five and three pollen specific elements are seen. Low temperature responsive elements are also present in the Gβ promoter. Similar cis-acting elements have been reported to be present in the promoters of low temperature responsive genes of Arabidopsis (rd29, cor15, kin1, kin2 and rab18; Nordin et al., 1993). The presence of light regulatory elements and tissue specific elements accounts for the tissue specific light regulation of Gα and Gβ. Three putative Dof binding sites are present in Gα and Gβ. Dof proteins are unique to plants and contain a highly conserved DNA binding domain that binds to a core AAAG sequence. In maize, Dof1 is constitutively expressed in roots, leaves and stem and acts as a transcriptional activator while Dof2 is expressed in roots and stem and acts as a transcriptional repressor (Yanagisawa and Sheen, 1998) for light mediated expression of C4 photosynthetic phosphoenolpyruvate carboxylase (C4PEPC). Thus Dof proteins may have tissue specific effects. DOF proteins have also been implicated in regulation of hormonal responses and pathogen attack (Yanagisawa, 2000). The light regulatory
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elements such as CCA1 have the sequence AAA/CAATCT and bind to myb-like factors (Wang et al., 1997). In most cases the myb binding site is found upstream of a CCAAT sequence. A general model of LRE function proposes that LREs are multipartite cis-regulatory elements with two general components: “light specific elements” and “coupling elements” (Arguello-Astorga and Herrara-Esterella, 1998). The former consists of the GATA/GT-1/I-box and are bound by transcription factors targeted by the light signal transduction pathway, which confer photoresponsiveness. The coupling elements are either bound by cell specific factors or regulatory proteins targeted by other signaling systems; consequently the light stimulus to transcription is coupled to other endogenous or exogenous signals. In this context the clustering of the GATA, GT-1 box, DOF binding site and the CCA1 box in a segment corresponding to the -400 to -650 region is significant. The exact interplay of factors that confer this light dependent tissue specific response seen for Ga and Gβ will only be understood by in vivo characterization of the promoter.

5.2 Localization of G proteins in plant cell

As shown in Fig. 2.2; 2.4 and 2.5, G proteins are attached to GPCR and residue inside of plasma membrane. Indeed in 1991, Warpeha et al. reported that a 40 kDa polypeptide, which is blue-light activated in the plasma membrane of etiolated pea, is recognized by polyclonal antisera directed against the α subunit of the G protein transducin (Warpeha et al., 1991). Recently the α and β subunits have been shown to be localized in the plasma membrane fraction in Arabidopsis (Weiss et al., 1997), rice (Iwasaki et al., 1997) and tobacco (Peskan and Oelmuller, 2000). This is reasonable because heterotrimeric G proteins must interact with receptor molecules present in the plasma membrane. In addition to the plasma membrane fraction, the α subunit was also found in the endoplasmic membrane fraction from Arabidopsis (Weiss et al., 1997), and the β subunit, in a purified nuclear fraction from tobacco (Peskan and Oelmuller, 2000).

Our data support the conclusion that both Ga and Gβ proteins are present in microsomal fraction (Fig. 8.14 and 8.38). Ga and Gβ were however also detected in chloroplast, cytosol, and nuclei fraction,. This is consistent with the report that G protein subunits have been also found in other cell fraction, where they might have other functions, like vesicle transport, the formation of the mitotic spindle and the golgi network, rough
endoplasmic reticulum, as well as protein transport (Schurmann et al., 1992; Pennington, 1995; Audiger et al., 1988; Weiss et al., 1997; Helms, 1995; Hutton, et al., 1998). Taken together, the distribution of the G proteins in various cellular compartments indicates that it not only mediates extracellular signals, but also intracellular signals. The size difference between recombinant with native protein was observed. The recombinant protein and the protein that produced by in vitro transcription and translation, were 44 kDa (Gα) and 41 kDa (Gβ) (Fig. 8.13 and 8.37), while the native protein were 40 kDa and 36 kDa respectively (Fig. 8.14 and 8.38). This lead us to suspect that G proteins are modified in plant cell after its translation.

5.3 Function of G proteins - transgenic approach

5.3.1 Function of Gα subunit

Transgenic rice containing antisense cDNA for the Gα subunit produced little or no mRNA for the subunit and exhibited abnormal morphology, including dwarf traits and the setting of small seeds (Fujisawa et al., 1999). This led to the discovery that the rice dwarf mutant, Daikoku d1, a traditionally famous mutant, has mutation in the RGA1 (rice G protein α subunit) gene (Ashikari et al., 1999; Fujisawa et al., 1999). Daikoku d1 shows abnormal morphology very similar to that of the transformants lacking RGA1 mRNA, and Daikoku d1 has been shown to be a gibberellin insensitive mutant (Mitsunaga et al., 1994, Ueguchi-Tanaka et al., 2000). In Arabidopsis, overexpression of Gα subunit by transgenic approach exhibited a hypersensitive response to light. This enhanced light sensitivity was more exaggerated in a relatively lower intensity of light and was observed in white light as well as far-red, red, and blue light conditions (Okamoto et al., 2001). gpa1 null mutants have reduced cell division in aerial tissues throughout development. Inducible overexpression of GPA1 in Arabidopsis confers inducible ectopic cell division. GPA1 overexpression in synchronized BY-2 cells cause premature advance of the nuclear cycle and the premature appearance of a division wall. Result from loss of function and ectopic expression and activation of GPA1 indicate that this subunit is a positive modulator of cell division in plants (Ullah et al., 2001)

In the present study, both α I and α II subunits antisense transgenic plants showed rooting delay, dwarfism, small pod, small seed size and faster aging. This suggests that G protein α subunit is involved in the development of normal seeds in tobacco. The sense
transgenic tobacco did not show obvious abnormal morphology. Hence, it is possible that antisense transcript did interact with endogenous gene causing repression of endogenous gene resulting in altered morphology.

If the Gα subunit is of fundamental importance in development of plant, the next question is to find out if two Gα subunits have the same function or not? According Marsh and Kaufman (1999), transcripts assay of PGA1 and PGA2 revealed that the expression of PGA1 and PGA2 mRNA is differentially regulated: PGA2 transcript is always present at a reduced level relative to PGA1 transcript. In yeast mating experiment, PGA1 negatively regulates the pheromone response, while both PGA1 and PGA2 promote growth independent of the mating pathway. As explained by authors, although the differential functions of PGA1 and PGA2 seem to be at odds with the striking degree of amino acid sequence homology between these two subunits, the dissimilarity in primary structure that exists between these α subunits occurs in precisely the region that are critical for receptor, Gβγ and effector interaction. Taken together with our transgenic plant data, lead us to conclude that PsGα I and PsGα II have the same role, but their expression level are different. The detailed functions of these subunits however is still remains unknown.

5.3.2 Function of Gβ subunit

Transgenic tobacco of both sense and antisense of Gβ were raised. It was found that both sense and antisense Gβ plants grow well. The antisense Gβ grow faster (from callus stage, Fig. 8.54) and taller than wild type (Fig.8.56). No abnormal morphology was observed in sense plants, except that some sense plants showed clustered leaves. While in Arabidopsis, Gβ null mutant, agb1-1 exhibits several defects, including short, blunt fruits, rounded leaves, and shortened floral buds (Lease et al., 2001).

The present evidence revealed that the function of Gα and Gβ are different, though they are two subunits of one trimer protein. Arabidopsis genome sequence revealed that there is only one Gα, one Gβ and two Gγ (possibly more) (Arabidopsis genome initiative, 2000; Assmann, 2002). Gα knock out showed reduced cell division (Ullah et al., 2001); Gβ knock out showed short, blunt fruit, rounded leaves, and shortened floral buds (Lease et al., 2001). In rice, Gα knock out showed dwarfism, dark green leaf, and small round
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seed. In the present work, both antisense of Gα and Gβ transgenic tobacco plants were raised, Gα antisense showed dwarfism, including small leaves, short stem in rooting stage, small pod, seed, and aging faster in mature stage, while Gβ antisense showed faster growth. It suggested that Gα subunit and Gβ subunit have different functions.

5.4 G proteins interact with PLC-δ in plant.
From the result of protein-protein interaction in vivo and in vitro, it was found that all G protein three subunits, α, β and γ interacts with PLC and its C2 domain. In animal system, Phopholipase C have four isoforms: β, γ, ε, and δ. They commonly have three domain: PH, X/Y TIM barrel and C2 domain. Gq interact with PLC-β have been reported (Exton, 1997). The site of interaction of Gαq with the PLC-β isozyme has been localized to its carboxyl terminus (Wu et al., 1993; Kim et al., 1996). Heterotrimeric G proteins are known to mediate PIP2 hydrolysis via PLC, and this effect is specific for Gq subfamily and PLC-β isozymes (Exton, 1997). A 42 kDa activated G protein α subunit was found to markedly stimulate PIP2 hydrolysis (Taylor et al., 1991), the βγ subunits of G protein have also been shown to modulate PLC-β activity (Singer et al., 1997). In contrast, in plants only δ-isofoms have been reported till now: Arabidopsis, soybean, potato and N. rustica (Hirayama et al., 1995; Yamamoto et al., 1995; Shi et al., 1995a; Kopka et al., 1998b), but report of G protein interacting with PLC is not available. Here, it is the first time demonstrated that the G proteins (all three subunits, not only α subunit) interact with PLC in plant.

5.5 G protein α, β and γ interact with each other
Recently plant Gγ was isolated and shown to interact with the plant β subunit by two different approaches, the two-hybrid method and in vitro translation experiment (Mason and Botella, 2000). However, no evidence has been presented for the interaction between the α subunit and the βγ dimers in plants. Since plant heterotrimeric G proteins have not yet been purified, there is no proof that a heterotrimeric G protein composed of the α, β and γ subunits is really present in plant cells.

In our present work, heterotrimeric G proteins, α, β and γ subunits were shown to interact with each other which was confirmed both in vitro and in vivo experiments.
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(yeast two hybrid system). Further β-galactosidase activity assay revealed that β and γ subunits have strong interaction, while α and β have weak interaction, the interaction can be classified as βγ>αγ>αβ. But in a previous report, Gα subunits, PGA1 and PGA2 did not interact with STE4 (Gβ in yeast) in the yeast two hybrid assay (Marsh and Kaufman, 1999). It seems that plant Gα does not interact with yeast Gβ, while Gα interacts with Gβ if they are cloned from same species, as for example in the present study where they both were cloned from pea.

Through the protein-protein interaction, a novel finding is that Gγ subunit can interact with PLC, and Gγ can form dimer. This leads us to explore the new function of Gγ subunit. The function of G protein, was mainly focused on α subunit, and βγ was studied as dimer, the sole function of Gγ is still unknown. Further work is needed to identify the function of Gγ by biochemical approach or transgenic approach, to understand the role of Gγ in signal transduction.