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RESEARCH ARTICLE

The physical interaction between LdPLCs and *Arabidopsis* G beta in a yeast two-hybrid system

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Abstract **Phosphoinositide-specific phospholipase C plays pivotal roles in a host of physiologic processes in both animals and plants. Animal PI-PLC is regulated by heterotrimeric G-protein. Plant PI-PLCs are structurally close to the mammalian PI-PLC-ζ isoform, and it is not testified what regulated this isoform enzyme. In this paper, two isoform genes of LdPLC (Pan, 2005) and three subunits of heterotrimeric G-protein in *Arabidopsis* were amplified and recombinated with plasmids of a yeast two-hybrid system. Using this system, we provided the evidence that LdPLC1 and Gβ subunit could be able to interact with each other. This result indicated that LdPLC1 might be regulated by G-protein.**

Keywords phosphoinositide-specific phospholipase C (PI-PLC), heterotrimeric G-protein, yeast two-hybrid

Introduction

Phosphoinositide-specific phospholipase C (PI-PLC) plays regulatory roles in a host of physiologic processes in both animals and plants. PI-PLC-mediated signaling has been proposed to be important in the plant response to various stimuli, including osmotic stress, ABA, light, gravity, pathogen attack, pollination and light-dependent phosphorylation of C4 phosphoenolpyruvate carboxylase for C4 photosynthesis (Chapman, 1998; Munnik et al.,1998; Perera et al., 1999; Coursol et al., 2000; Perera et al., 2001; Sanchez and Chua, 2001). A recent report suggests that PI-PLC isoforms are required for the hypersensitive response and disease resistance in tomato (Vossen et al., 2010). Tasma et al. (2008) have studied the expression patterns of all nine *AtPLC* genes in different organs and in response to various environmental stimuli by applying a quantitative RT-PCR approach, finding that multiple members of the gene family are differentially expressed in *Arabidopsis* organs, and suggesting putative roles for this enzyme in plant development, including tissue and organ differentiation. This study also shows that a majority of the *AtPLC* genes are induced in response to various environmental stimuli, including cold, salt, nutrients Murashige-Skoog salts, dehydration, and the plant hormone abscisic acid. There are also many evidences about the function of PI-PLC in the plant pollen system. Helsper, Heemskerk and Veerkamp (1987) first demonstrated the presence of PI-PLC activity in pollen tubes of *Lilium longiflorum*, while Franklin-Tong et al. (1996) provided a pharmacological evidence for the presence of a Ca^{2+}-dependent PI-PLC activity in *P. rhoeas* pollen, indicating the correlation between PI-PLC activity and pollen tube growth during the self-incompatibility process. Recent evidences suggest that *petunia* PLC1 is involved in pollen tube growth (Dowd et al., 2006), and the pollen tube tip growth depends on plasma membrane polarization mediated by tobacco PLC3 activity (Helling et al., 2006).

PI-PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate and generates inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, both of which are second messengers in the phosphoinositide signal transduction pathways operative in animal and plant cells (Meijer and Munnik, 2003). Five PI-PLC isoforms, β, γ, δ, ε and ζ, have been identified in mammals. The enzymatic activities of each type of PI-PLC are regulated by distinct mechanisms. PI-PLC-γ is activated by phosphorylation of tyrosine residues by receptors with intrinsic tyrosine kinase activity or by nonreceptor tyrosine kinases, while others are regulated by the heterotrimeric G protein subfamily. Plant PI-PLCs are structurally close to the mammalian PI-PLC-ζ isoform. Regulation of PI-PLC-ζ is still unknown; some evidences suggest that plant PI-PLCs might

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be regulated by G protein (Meijer and Munnik, 2003). Our previous work isolated two full-length PI-PLC cDNAs from *Lilium daviddi* pollen, detected their PIP2-hydrolyzing activity, and provided evidences that the PI-PLC activity is present and might be regulated by heterotrimeric G-protein and exogenous CaM in pollen (Pan et al., 2005). Here we detected if LdPLCs interacted with G protein by a yeast two-hybrid system.

Materials and methods

Yeast strain, transformation, and growth

Saccharomyces cerevisiae strain Y187 was used in our study. The partial genotypes of Y187 are MAT, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4, met-, gal80, URA3: GAL1UAS-GAL1TATA-lacZ, MEL1. Y187 harbors the LacZ reporter gene. Yeast transformation was performed by using a lithium acetate-based protocol (Ashikari et al., 1999). Standard media were used for growth (Ueguchi-Tanaka et al., 2000). Glucose was used as the carbon source for transformation, and sucrose was used as the carbon source for the two-hybrid assay. Yeast strains, vectors, reagents, and methods were adapted from the BD MATCHMAKERTM Library construction & Screening Kit (Clontech, Palo Alto, California, USA).

PCR amplification of GPA1, AGB1, AGG1 and LdPLC1/2 ORFs

PCR primers for each ORF (Table 1) constructed from Sangon Biotech (Shanghai) Co., Ltd were used for amplification from *Arabidopsis* (Gα/Gβ/ Gγ) or *Lily* (LdPLC1/ LdPLC2) cDNA. The PCR conditions of three subunits of G protein were at 94°C for 5 min; 94°C 30 s, 58°C 30 s, 72°C 1.5 min, 30 cycles; 72°C 10 min. The PCR conditions of LdPLCs were at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 1.5 min, with final extension at 72°C for 10 min. Primers to add restriction enzyme sites to both ends of each ORF (Gα was *Nde*I and *Eco*RI, Gβ,Gγ and LdPLCs were *Nde*I and *Bam*H) were digested with the enzymes and used for the cloning into the two-hybrid vectors described below. The sequences of each ORF were measured by Sangon Biotech (Shanghai) Co., Ltd. Polymerase and reagents were from TAKALA. The bacterial strain *Escherichia coli* DH5α was used for the propagation of the plasmid constructs.

Construction of two-hybrid vectors

For a GAL4 activation domain-fusion vector, the amplified ORF fragments of Gβ, Gγ, LdPLC1 and LdPLC2 were respectively inserted into a multiple cloning site between the *Bam*HI site and the *Nde*I and *Eco*RI site of pGADT7, which carries the *Ampr* gene. Similarly, a GAL4 DNA binding domain fusion vector pGBKT7-Gα (Gβ,Gγ) was constructed by inserting the ORF fragments of the Gα (Gβ,Gγ) into the multiple cloning site between the *Bam*HI, *Nde*I and *Eco*RI sites of pGBKT7, which carries the *Kanr* gene (Table 2).

Yeast two-hybrid assay

Yeast strain Y187 was transformed simultaneously with the recombine plasmid listed in Table 2, and each yeast strain cotransformed was named by the ORF in the plasmid (Table 2). The ability to drive the expression of yeast *HIS3* reporter gene was tested by growing transformants on the selective medium lacking tryptophan, leucine, and histidine. *LacZ* reporter gene activity in the yeast cells was monitored visually by the 5-bromo-4-chloro-3-indolyl-b-D-galactopy ranoside (X-Gal) filter assay for the X-Gal filter assay according to yeast protocol handbook PT3024-1 (PR13103).

Table 1 PCR primer sets for the amplification of each ORF

name of ORFs	gene	primer	primers	references
Gα	GPA1	forward primer	5′ ggaattccatatgggcttactctgcagtag 3′	Ma et al., 1990
		reverse primer	5′ cggaattctcataaaaggccagcctccag 3′	
Gβ	AGB1	forward primer	5′ ggaattccatatgtctgtctccgagctc 3′	Weiss et al., 1993
		reverse primer	5′ cgggatcctcaaatcactctcctgtgtc 3′	
Gγ	AGG1	forward primer	5′ggaattccatatggaagcgggtagctcc 3′	Mason and Botela, 2001
		reverse primer	5′ cgggatcctcaaagaatggagcagcc 3′	
LdPLC1	*LdPLC1*	forward primer	5′ ggaattccatatgaatgccccgctgtcacac 3′	Pan et al., 2005
		reverse primer	5′ cgggatccctatatatatacatagggattacaaactatc 3′	
LdPLC2	*LdPLC2*	forward primer	5′ ggaattccatatgctgagcggattgtcgatcag 3′	
		reverse primer	5′ cgggatcctcactcaatttctacagaactaaagc 3′	

Table 2 Name of the cotransformed yeast strain and recombine plasmid

name of the co–transformed yeast strain	recombined plasmid of pGBKT7	recombined plasmid of pGADT7
αβ	pGBKT7-Gα	pGADT7-Gβ
αγ	pGBKT7-Gα	pGADT7-Gγ
βγ	pGBKT7-Gβ	pGADT7-Gγ
α0	pGBKT7-Gα	pGADT7
β0	pGBKT7-Gβ	pGADT7
γ0	pGBKT7-Gγ	pGADT7
αL1	pGBKT7-Gα	pGADT7-LdPLC1
βL1	pGBKT7-Gβ	pGADT7-LdPLC1
γL1	pGBKT7-Gγ	pGADT7-LdPLC1
αL2	pGBKT7-Gα	pGADT7-LdPLC2
βL2	pGBKT7-Gβ	pGADT7-LdPLC2
γL2	pGBKT7-Gγ	pGADT7-LdPLC2

Results

Plasmid constructions

To detect the interaction between Gα/β/γ and LdPLCs, we constructed the subunit Gα/β/γ into plasmids DB and AD, respectively. We amplified the ORF of Gα/β/γ subunits by PCR, and the PCR products were confirmed by sequencing, and then recombined into pGBKT7 and pGADT7, respectively (Fig. 1). In the same way, LdPLCs were recombined into pGADT7, respectively (Fig. 2). Figures 1 and 2 show the results of PCR amplification of each ORF and restriction analysis of the recombined plasmid.

Growth on SD/-Trp-Leu-His-Ade medium and X-gal filter assay

Autonomous activation assay of G-protein α/β/γ subunits
To detect the autonomous activation effect of pGBKT7-Gα, pGBKT7-Gβ and pGBKT7-Gγ, we co-transformed the pGBKT7-Gα, pGBKT7-Gβ and pGBKT7-Gγ with plasmid AD into the yeast strain Y187, respectively, by using the LiAc transformation method (Yeast Protocols Handbook). The results (Table 3 and Fig. 3) showed that α0, β0 and γ0 were able to grow on the synthetic dropout nutrient medium (SD/-Trp-Leu), and that the plasmid combinations had transformed into Y187, respectively; however, they were not able to grow when inoculated on the synthetic dropout nutrient medium (SD/-Trp-Leu-His-Ade), and the X-gal filter of the colony was not turning blue, which suggested that the three subunits of G protein were not able to activate downstream reporter gene. Therefore, they can be used in the interaction analysis with other proteins as a negative control.

The interaction analysis between three subunits of G protein
We detected the interaction between the three G protein subunits by the same method, the combination of co-transformation and the growth situation on the synthetic dropout nutrient medium (SD/-Trp-Leu-His-Ade), and x-gal filter results were shown in Table 2 and Fig. 4. The results

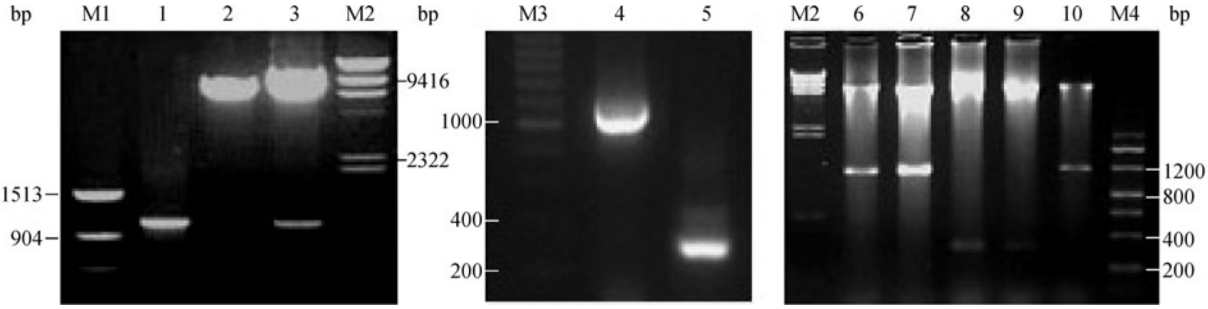

Figure 1 PCR amplification of G-protein α/β/γ subunits and restriction analysis of the recombined plasmid
Note: M1, M3 and M4 represent PCR markers. M2 represents λDNA/*Hind*III markers. 1–10 represent Gα PCR products, pGBKT7/*Nde*I + *EcoRI*, pGBKT7-Gα/*Nde*I + *Eco*RI, GβPCR products, Gγ PCR products, pGADT7-Gβ/ *Nde*I + *Bam*H, pGBKT7-Gβ/ *Nde*I + *Bam*HI, pGADT7-Gγ/ *Nde*I + *Bam*H, pGBKT7-Gγ/ *Nde*I + *Bam*H and pGBKT7-Gα/ *Nde*I + *Eco*RI, respectively.

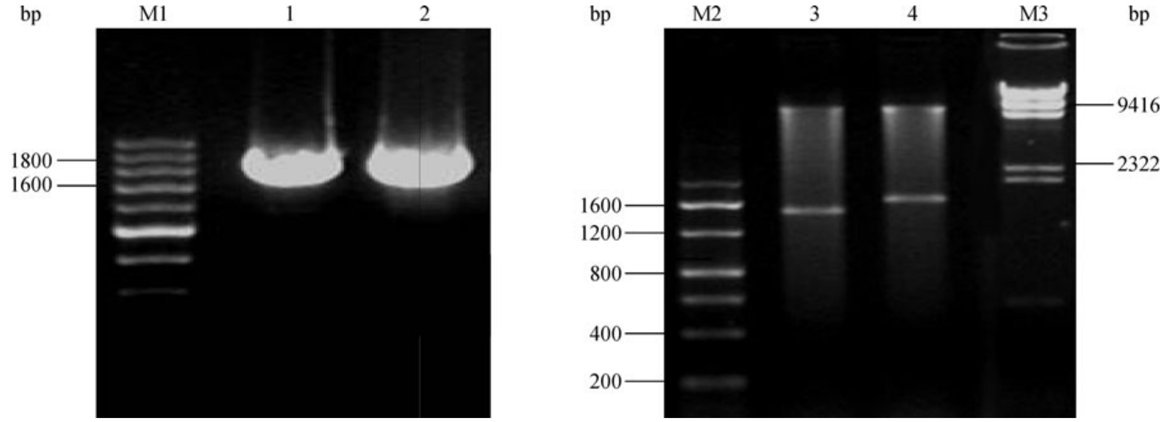

Figure 2 Restriction analysis of plasmids recombined with LdPLCs and pGADT7
Note: M1 and M2 are PCR markers. M3 is λ DNA/*Hin*dIII marker. 1–4 are LdPLC1 PCR products, LdPLC2 PCR products, pGADT7-LdPLC1 / *Nde*I + *Bam*H and pGADT7-LdPLC2/ *Nde*I + *Bam*HI, respectively.

Table 3 Autonomous activation analysis of three G–protein subunits

name of the co–transformed yeast strain	plasmid combination	growth on SD/–Trp–Leu medium	growth on SD /–Trp–Leu–His–Ade medium	X–gal filter analysis
α0	pGBKT7–Gα + pGADT7	+	–	–
β0	pGBKT7–Gβ + pGADT7	+	–	–
γ0	pGBKT7–Gγ + pGADT7	+	–	–

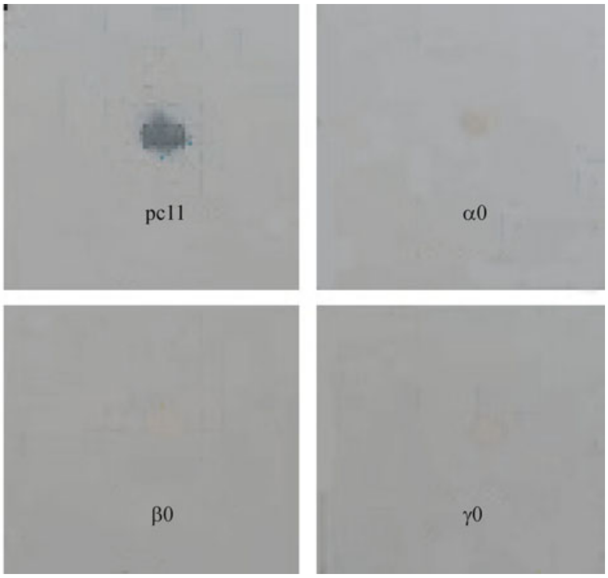

Figure 3 Autonomous activation assay of G-protein α, β and γ subunits
Note: x-gal filter results of α0, β0 and γ0 from SD/-Trp-Leu medium are negative and pc11 is positive control.

showed that the combination βγ was able to grow on the synthetic dropout nutrient medium (SD/-Trp-Leu-His-Ade) with positive x-gal filter result, which indicated that there existed a strong interaction between β and γ. αβ could grow on the synthetic dropout nutrient medium (SD/-Trp-Leu-His-Ade), but the result of x-gal filter was negative, which implied that there was a weak or instantaneous interaction between α and β. These results were made as a positive control to measure the interaction between Gα/Gβ/Gγ and PLC. αγ was not able to grow on the synthetic dropout nutrient medium (SD/-Trp-Leu-His-Ade), which suggested no interaction between α and γ.

The interaction analysis of Gα/Gβ/Gγ subunits with LdPLCs
On the basis of experiments above, we detected the interaction between the Gα/Gβ/Gγ and LdPLCs, and listed the combination of possible interaction in Table 5 and Fig. 5. The results exhibited that αL2, αL1, γL2 and γL1 cannot grow on the synthetic dropout nutrient medium (SD/-Trp-Leu-His-Ade), and the X-gal filter results of colony growing on SD medium (SD/-Trp-Leu) were also negative, suggesting that the subunits α and γ were not able to interact with LdPLCs; βL2 was able to grow on the SD medium (SD/-Trp-Leu-His-Ade), but still with negative x-gal filter, which indicated that there was a weak interaction between LdPLC2 and subunit β, just like the αβ (Table 4, Fig. 4). βL1 was able to grow on the SD medium (SD/-Trp-Leu-His-Ade), with a positive X-gal filter, which indicated that LdPLC1 and subunit β were able to interact with each other, like the βγ (Table 4, Fig. 4).

Table 4 The interaction analysis between the Gα/β/γ subunits

name of the co–transformed yeast strain	plasmid combination	growth on SD/–Trp–Leu medium	growth on SD /–Trp–Leu–His–Ade medium	X–gal filter analysis
αβ	pGBKT7–Gα + pGADT7–Gβ	+	+	−
αγ	pGBKT7–Gα + Pgadt7–Gγ	+	−	−
βγ	pGBKT7–Gβ + pGADT7–Gγ	+	+	+
γβ	pGBKT7–Gγ + pGADT7–Gβ	+	+	+

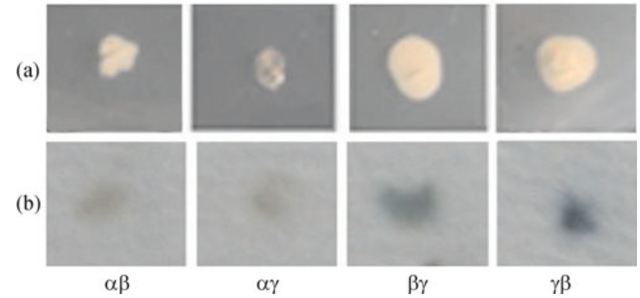

Figure 4 The interaction analysis of G-protein α/β/γ subunits
Note: A represents growth on SD medium (SD/-Trp-Leu-His-Ade) and B represents x-gal filter analysis.

Discussion

The heterotrimeric G-proteins act as critical molecular switches, and signaling through G-proteins is a conserved mechanism found in all eukaryotes. In plants, the repertoire of G-protein signaling complex is much simpler than that in metazoans (Chen, 2008). The genome of *Arabidopsis* encodes only one canonical Gα, one Gβ, and two Gγ subunits (Ma et al., 1990; Weiss et al., 1993; Mason and Botela, 2000; 2001). The analyses of loss-of-function alleles and gain-of-function transgenic lines of G-protein subunits suggest that the three subunits of G-protein all play regulatory roles in multiple developmental processes. Gα and Gβ are viewed as regulators of GA and BL signaling in seed germination of *Arabidopsis thaliana* and rice (Ashikari et al., 1999; Ueguchi-Tanaka et al., 2000; Ullah et al., 2002; Chen et al., 2004; Trusov et al., 2007). GPA1 and AGB1 (Gβ subunit) single and double mutants are hypersensitive to ABA in seed germination assays (Ullah et al., 2002; Pandey et al., 2006). AGG1 and AGG2 (two Gγsubunits) single and double mutants are hypersensitive to high concentrations of dglucose or mannitol (Ullah et al., 2002; Pandey et al., 2006). G protein α and β subunits antagonistically modulate stomatal density in *Arabidopsis thaliana* (Zhang et al., 2008). In addition, G-proteins are also involved in early seedling development (Chen et al., 2004; Pandey et al., 2006; Misra et al., 2007;), root development (Pandey et al., 2006), organ shape determination (Misra et al., 2007; Chen et al., 2004; Oki et al., 2005; Peškan-Berghöfer et al., 2005), cell division (Chen et al., 2006) and extCaM-induced stomatal closure by activated H_2O_2 production and NO accumulation (Li et al., 2009). Recent reports show that GPA1 (G subunit) is a regulator of transpiration efficiency (Nilson and Assmann, 2010).

Gα, β, γ were also identified in many other plants, but these subunits are predicted to form only two possible G-protein heterotrimers in *Arabidopsis thaliana* or rice (Chen, 2008). AGG1 and AGG2 were identified from a yeast two-hybrid screen in which AGB1 was used as bait (Mason and Botela, 2001). The assembly of the GPA1-AGB1-AGG1/AGG2 has been demonstrated using fluorescence resonance energy transfer (FRET) imaging (Li et al., 2009). The physical interaction between three subunits of G proteins has been confirmed at both the biochemical and cellular levels (Chen, 2008). We further confirmed the physical interactions between GPA1 and AGB1, AGB1 and AGG1 by a yeast two-hybrid system in this article. Therefore, our data of interactions between G proteins and LdPLCs were also assured. In our experiment, although αβ grew on the SD medium (SD/-Trp-Leu-His-Ade), they showed a negative dyeing result which suggested that there was a weak interaction between them (Table 4, Fig. 4). However, the dyeing result of βγ and γβ was positive, showing that these combinations were more stable and fitted the present regulation modes of G-proteins. According to the above control experiments, LdPLCs had no interaction with Gα, LdPLC2 had weak interactions with Gβ and LdPLC1 had strong interactions with Gβ. These results indicated that LdPLC1 was regulated by G-protein, which was identical with our previous work (Pan et al., 2005).

In the classical model of G-protein signaling, the G-proteins receive input signals from upstream 7TM GPCRs and act through downstream effector proteins. Four proteins, including AtPIRIN1, THF1, PD1and PLDα1, have shown their physical interactions with *Arabidopsis* Gα and GPA1, thus candidate downstream effectors for GPA1. Three of these four proteins were identified in yeast two-hybrid screens (Chen, 2008). So far, the only protein, SGB1, a golgi-localized hexose transporter, has been shown to be genetically coupled to *Arabidopsis* Gβ (AGB1) when regulating cell division in the hypocotyls and sugar sensing except Gα and Gγ (Adjobo-Hermans et al., 2006; Wang el at., 2006). Misra et al. (2007) reported that the transcript levels of Gα and Gβ from *Pisum sativum* were upregulated following NaCl, heat and H_2O_2 treatments. In addition, they provided evidences for

Table 5 The interaction analysis of Gα/Gβ/Gγ subunits with LdPLCs

name of yeast stain	plasmid recombination	growth on SD /–Trp–Leu medium	growth on SD /–Trp–Leu–His–Ade medium	X–gal filter analysis
αL2	pGADT7–Gα + pGBKT7–LdPLC2	+	–	–
αL1	pGADT7–Gα + pGBKT7–LdPLC1	+	–	–
βL2	pGADT7–Gβ + pGBKT7–LdPLC2	+	+	–
βL1	pGADT7–G + pGBKT7–LdPLC1	+	+	+
γL2	pGADT7–G + pGBKT7–LdPLC2	+	–	–
γL1	pGADT7–G + pGBKT7–LdPLC1	+	–	–

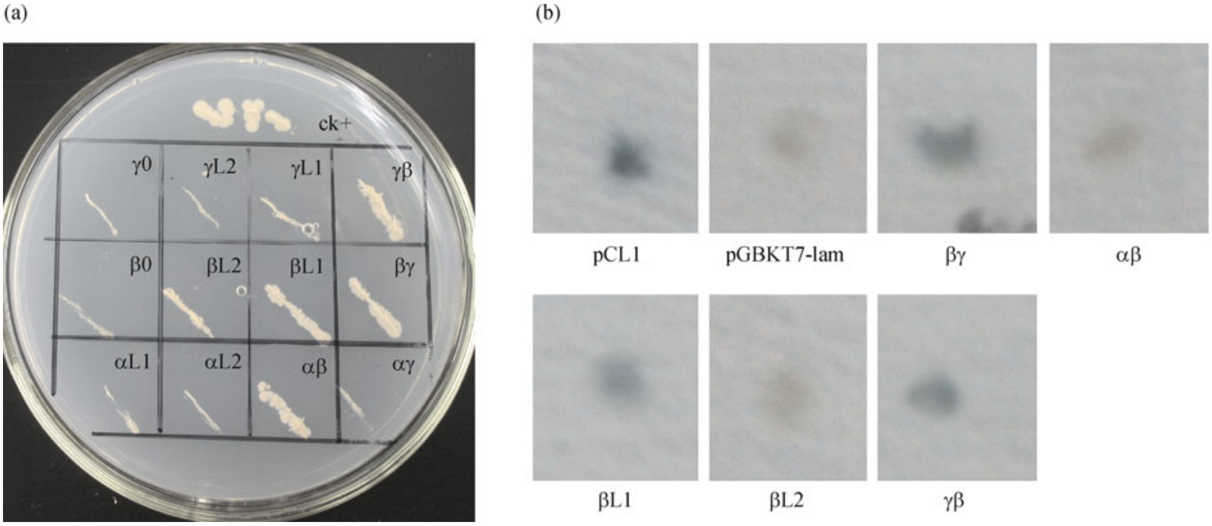

Figure 5 The growth of the co-transformated yeast clones on SD/-Trp-Leu-His-Ade medium (a) and their β-gal assay (b)

using the yeast two-hybrid system, and in plant, the co-immunoprecipitation showed that the Gα subunit interacted with the pea Gβ subunit and pea phospholipase C (PLCδ). Our result in this paper showed that the LdPLCs might be also coupled to Gβ (Table 5 and Fig. 5) and regulated by G protein in pollen germination (Pan et al., 2005).

Though it was rarely examined that plants PI-PLC could be coupled to Gβ, there was no lack of the evidences about interaction of PI-PLC with Gβ or Gα in animals. The results from Wing et al. (2001) revealed that the presence of additional functional domains in PLC-ε increased a new level of complexity in the regulation of this novel enzyme by heterotrimeric G proteins. Kowalczyk and Hetmann (2008) showed indirect evidences and suggested that Gα may interact with ion channels and phospholipases A2 and C, whereas Gβγ dimer supposedly interacts with a Golgi-localized hexose transporter. Zhou et al. (2008) illustrated that PLC-eta2 was a direct downstream effector of Gβγ and, therefore, was also that of receptor-activated heterotrimeric G proteins. Gβ3 can form a distinct dimer with specific Gγ subunits and preferentially activate the β3 isoform of phospholipase C (Poon et al., 2009). The prediction of protein–protein interfaces on G-protein β subunits reveals a novel phospholipase C β2 binding domain (Friedman et al., 2009). Although there are only a few subunits in G proteins of plants, the PLC isoforms are a large family; the study of their physiologic function and the position of signaling pathway is of the greatest importance, which indicates that it is propitious to open out molecular mechanisms of plant responses to abiotic stresses.

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