Cell signalling in Paracoccidioides Brasiliensis

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CELL SIGNALLING IN

PARACOCCIDIOIDES BRASILIENSIS

by

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Paracocidioides brasiliensis (*P. brasiliensis* or *Pb*) is the etiological agent of paracocidioidomycosis which is the most prevalent systemic mycosis in South America. About 60% of the clinical cases are found concentrated in Brazil and the disease is a major threat for the public health there. *P. brasiliensis* is a dimorphic fungus because it undergoes a morphological switching from a mycelium to yeast form after shifting the temperature from 26°C to 37°C. Similar morphological changes have been implicated to be important in the pathogenicity of other dimorphic fungi and they appear to be under the control of the cAMP signalling transduction pathway.

In order to establish the relationship of cAMP signalling pathway and the morphological change in *P. brasiliensis*, a project was initiated to clone the key components of the cAMP signalling pathway and analyze their functions. To this end, a genomic DNA library and two cDNA libraries of *Pb* were constructed. Degenerate primers were synthesized based on the sequence of genes of several other important fungi in GenBank. After amplification, specific PCR products were obtained and sequenced. The specific PCR products were used for labeling to screen libraries and

their sequences were used for designing specific primers to do genomic walking and RACE-PCR. Both cDNA and genomic DNA sequences have been determined for the key components of the cAMP signalling pathway including adenylate cyclase (*PbCYR1*), three G protein α subunits (*PbGPA1*, *PbGPA2* and *PbGPA3*), a G protein β subunit (*PbGPB1*), a G protein γ subunit (*PbGPG1*), a cAMP dependent protein kinase catalytic subunit (*PbTPK1*), a cAMP depentdent protein kinase-like gene (*PbTPKL1*) and a *TUP* gene (*PbTUPA*).

The interactions of the proteins encoded by the genes in the cAMP signaling pathway were studied with Clontech's Matchmaker yeast-two-hybrid System III. This approach demonstrated that the N-terminal third of adenylate cyclase PbCyr1$^{1\text{-}678}$ can interact with PbGpa1 and PbGpb1. To further test if PbGpa2, PbGpa3, PbRas1, and PbActin can interact with adenylate cyclase, random mutagenesis libraries were made for these genes and screened with PbCyr1$^{1\text{-}678}$, PbCyr1$^{600\text{-}1316}$, PbCyr1$^{1302\text{-}1876}$ and PbCyr1$^{1648\text{-}2100}$. It was demonstrated that PbCyr1$^{1\text{-}678}$, where the Ras association domain resides, can interact with truncated versions of PbGpa2, PbGpa3, PbRas1, and PbActin, i.e., PbGpa2$^{1\text{-}102}$, PbGpa2$^{1\text{-}183}$, PbGpa3$^{1\text{-}160}$, PbGpa3$^{1\text{-}213}$, PbRas1$^{1\text{-}83}$, PbRas1$^{26\text{-}238}$, and PbActin$^{1\text{-}314}$.

For the first time, the major components of *Pb* cAMP signaling pathway have been cloned and characterized; and we provide direct evidence that the G protein α subunits, G protein β subunit, RAS protein and actin interact directly with adenylate cyclase in fungal biology. This thesis funds the basis for the further study of the cAMP signaling pathway in *P. brasiliensis*. It may facilitate delineation of the mechanism for the dimorphic switching and the development of potentially novel antifungal drugs.

Declaration

I declare that this thesis represents my own work, except where due acknowledgment is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University or to any other institution for a degree, diploma or other qualification.

Signed  .....

Daliang Chen

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Abbreviations

CAP	Cyclase-Associated Protein
CID	Centre for Infectious Diseases (Durham University)
CYCc	Adenylyl/guanylyl Cyclase, Catalytic domain
DTH	Delayed T-cell Hypersensitivity
EST	Expressed Sequence Tag
EtBr	Ethidium Bromide
FRE	Filamentation and invasion Response Element
GAP	GTPase-Activating Protein
GEF	Guanine Exchange Factor
GIPC	Glycosylinositol Phosphorylceramides
GPI	Glycosyl-Phosphatidyl-Inositol
GPR	G Protein-coupled Receptor
GPCR	G Protein-Coupled Receptor
GSL	Glycosphingolipid
HSP	Heat Shock Protein
IBLS	Institute of Biological and Life Science (University of Glasgow)
IPTG	Isopropyl-1-thio-β-D-galactoside
KRH	Kelch Repeat Homologue
LRR	Leucine-Rich Repeats
MAPK	Mitogen Activated Protein Kinase
MMcM	Modified McVeigh-Morton
MW	Molecular Weight
OD	Optical Density
Pb	*Paracoccidioides brasiliensis*
Pb01	*Paracoccidioides brasiliensis* strain ATCC 90659
PCM	Paracoccidioidomycosis
PCR	Polymerase Chain Reaction
pfu	Plaque Forming Units
PKA	cAMP-dependent Protein Kinase
PP2Cc	Protein serine/threonine Phosphatase 2C, catalytic domain
PRE	Pheromone Response Element
RA	Ras Association
RACE	Rapid Amplification of cDNA End
RM	Random Mutagenesis
rpm	Rounds per minute
RT	Reverse Transcription
SCAM	Substituted Cysteine Accessibility Method
SH3	Src Homology 3
SMART	Switching Mechanism At 5' end of RNA Transcript
SSP-PCR	Single Specific Primer PCR
TAE	Tris Acetate EDTA

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Chapter I

Introduction

1.1 General Introduction: *P. brasiliensis* and Paracoccidioidomycosis

Paracoccidioides brasiliensis (*P. brasiliensis* or *Pb*) is a fatal human pathogenic fungus that causes the disease paracoccidioidomycosis (PCM), which is geographically restricted to South and Central America (Borges-Walmsley et al, 2002). The fungus is dimorphic growing in a mycelial form at temperatures below 30°C (typically 26°C; Brummer et al, 1993), whilst in the human body, or on enriched media at 37°C, growing as a yeast form with a typical morphology described as "ship-pilot's wheel", a large globose cell surrounded with multiple daughter buds (Borges-Walmsley et al, 2002). The mycelia in the environment can produce conidia, which act as infectious propagules, causing infection by human inhalation. Conidia transform into the pathogenic yeast form after entry into the human body. Mammalian oestrogens inhibit the transition from conidia to yeast, giving rise to a much higher incidence of overt clinical manifestations in males (San-Blas, 1993; Hogan et al, 1996). The disease is always initiated from a pulmonary infection, which is usually benign and transient in normal individuals but can be latent for 10 years or more. The later reactivation results in a chronic lung infection that can disseminate to the mucosa of mouth, the nose, skin, adrenals, lymph nodes, and other organs. Susceptible individuals supposedly have a genetic basis with a depressed cellular immune response after infection (Borges-Walmsley et al, 2002). Currently, ketoconazole and itraconazole are the drugs of choice and treatment usually lasts 0.5-2 years (Brummer et al, 1993; Borges-Walmsley et al, 2002). Early diagnosis is important for prompt treatment and a better prognosis; because mortality is high if the disease is not treated. Relapse is a common problem after treatment.

Although the disease can be controlled by antifungal drugs, fibrotic sequelae in the lungs always constitute a major problem in the well-being of the patients for the rest of their life (Borges-Walmsley et al, 2002).

1.2 Morphology, Structure and Physiology of *P. brasiliensis*

P. brasiliensis is a dimorphic fungus. Dimorphism is a phenomenon in some fungi that can reversibly change between yeast and mycelial forms and it is frequently related to the virulence of these fungi. *P. brasiliensis* grows as a mycelial form at temperatures lower than 30°C (typically 26°C); and at 37°C, it grows as a yeast form in culture or in host tissue. A change of temperature is the only factor required for this dimorphic transformation (San-Blas, 1993). In cultures, the mold phase grows slowly, and it takes about 20-30 days for the mold colonies to become visible. Mold colonies are small, irregular and white to tan in color. The yeast phase grows slowly as well but faster than the mold form. Yeast colonies become apparent after 10-15 days of incubation. Yeast colonies are soft, creased, and cream colored (Brummer et al, 1993). The full transition from mycelia to yeast usually takes 3 weeks after the temperature change (Borges-Walmsley et al, 2002).

Microscopically, yeast cells are of different sizes from 4 to 30 μm (Brummer et al, 1993). The shapes of yeast cells may be round, oval, or elongated. The most characteristic feature of the yeast cell is the ship-pilot's wheel appearance, i.e. a multiple budding mother cell surrounded by several peripheral daughter cells, on which cultural and histological diagnosis are made. The ultrastructure of yeast cells includes 2 sublayers of cell wall, 3 sublayers of plasma membrane, large numbers of mitochondria, a scanty endoplasmic reticulum, numerous vacuoles and multiple

nuclei. The hyphae of mold under the microscope are thin and septate. Chlamydospores and conidia can be seen in some laboratory media (Brummer et al, 1993).

Under adverse environmental conditions such as a lack of water and low levels of nutrient and oxygen, two forms of propagules, i.e. chlamydospores and conidia, can be produced from mycelia (San-Blas, 1986). Both forms of propagule can reproduce the mycelia or yeast parent structure under appropriate incubation. Chlamydospores are multinucleated, while conidia are uninucleated. The formation of conidia is considered to be a terminal event under environmental stress. It is supposed that conidia exist in the environment and are the form to cause infection through inhalation by human beings. Their small size (3.5-5.0 μm) is compatible with alveolar deposition (San-Blas, 1986).

Cytochemical studies of both the yeast and mycelial cells focused on the cell wall components (Paris et al, 1986; Davilla et al, 1986). *In vivo*, α-1,3-glucan is the main polysaccharide in the yeast cell wall, and there are only traces of β-1,3-glucan. In contrast, β-1,3-glucan is the only form of glucan in the mycelial cell wall. The predominance of α-1,3-glucan in the yeast cell wall may have a role in protection against host defense because phagocytic cells do not digest α-1,3-glucan (Goldman et al, 2003), while β-1,3-glucan can be digested and trigger the inflammatory response of the host lungs (Vassallo et al, 2000). In addition, *Pb* strains containing less α-1,3-glucan and more β-1,3-glucan are avirulent (Silva et al, 1994), supporting the role of α-1,3-glucan in avoiding the inflammatory response.

Notably, there is an increase in chitin content during mycelia to yeast transformation (Borges-Walmsley et al, 2002). *Pb* has been shown to have several chitin synthesis genes but no correlation has been found between transcription levels of these genes and the increase in chitin content during the transition (Nino-Vega et al, 2000). The morphological change of *Pb* is also accompanied by changes in the lipid composition of the cell membrane. The total lipid extracted from yeast is twice as much as from mycelia (Manocha, 1980). In addition, the percentage of individual lipid components changes during the phase transition, especially for glycosphingolipids (GSLs) (Toledo et al, 1995; Levery et al, 1998; Toledo, et al, 1999). The major neutral GSLs are two forms of β-glucopyranosylceramides (GlcCer): unsaturated and saturated. The mycelia have both forms of GlcCer in a 1:1 ratio; while in yeast the saturated GlcCer predominates (85%; Toledo et al, 1999). Two major acidic GSLs were extracted and structurally analyzed. They were found to be glycosylinositol phosphorylceramides (GIPCs) that differed in that one contained a β-galactofuranose. The mycelial membranes contain equivalent amounts of GIPCs. The yeast membranes predominantly comprise the β-galactofuranose-containing GIPC which was reactive with sera from patients with PCM (Levery et al, 1998). The extract of GIPC without the β-galactofuranose was not reactive with the patients' sera. The immunological reactivity of the β-galactofuranose-containing GIPC was attributed mainly to the galactofuranosyl residue (Toledo et al, 1995). It was proposed that these changes in the composition of the cell membrane are used to regulate the synthases (e.g. glucan and chitin synthases) and hydrolases (e.g. glucnases and chitinases), which in turn modify the composition of the cell wall (Borges-Walmsley et al, 2002).

Another two chemical components correlated with pathogenicity are melanins and glycoprotein gp43 (Gomez et al, 2001; Vicentini et al, 1994; Hanna et al, 2000). Melanins play an important role in the virulence of the human pathogenic fungus *Cryptococcus neoformans* (Wang et al, 1995). It has been demonstrated that melanins protect the fungus from environmental insults, host defense and antimicrobial therapies. Melanins are found in *Pb* conidia and yeast cells and they are supposed to be a virulence factor for *Pb* as well (Gomez et al, 2001). The gp43 is a cell surface and exocellular glycoprotein with proteinase activity at acidic pH (Brummer et al, 1993). It is the major diagnostic antigen of *Pb* and this will be discussed later. In addition, it also acts as a virulence factor. It is a laminin receptor and responsible for the adhesion of *Pb* cells to the host cells: gp43 antiserum can abolish this binding activity (Hanna et al, 2000). The gp43 was also proposed to be responsible for invasion into host cells expressing laminin receptors. A hamster model indicated that *Pb* cells pre-coated with laminin enhance their virulence (Viceniti et al, 1994).

A sufficient supply of oxygen is necessary for the growth of both the yeast and mycelial phase of *Pb* (Restrepo et al, 1981). However, under microaerophilic conditions, yeast cells can enter into a resting stage. Resting cells can regain activity when adequate oxygen is supplied. This capacity of yeast cells may well explain the prolonged latency of this mycosis. As for nutritional requirement, the mycelia form of the fungus is prototrophic. However, yeast cells were shown to require an accessory factor for growth, i.e. a sulfur-containing amino acid (Paris et al, 1985).

The growth curve of the yeast phase has been studied: the doubling time of yeast

varied from 21 to 102 hours (Kashino et al, 1987). Many factors such as the strain of the fungus, culture media used and shaking speed can all affect the mean generation time. Growth curves do not correlate with the virulence of the fungus (Kashino et al, 1987). A chemically defined medium, MMcM (modified McVeigh-Morton), was used for growth of *Pb* yeast cells (Restrepo and Jimenez, 1980). Yeast cells in this liquid medium reached stationary phase after 3 days if the inoculation was from MMcM agar after 6 passages. In this situation, the doubling time of *Pb* in the log phase is only 1-2 hours.

1.3 Epidemiology

Paracoccidioidomycosis (PCM) is restricted to Latin America, from Mexico (23°N) to Argentina (34°S; Brummer et al, 1993). The disease is not evenly distributed in this scope of land. There are no reported cases in the Caribbean islands, the Guyanas, Surinan, Chili or Nicaragua. Brazil is the most affected country, accounting for 80% of reported cases, followed by Colombia and Venezuela. The so-called endemic areas are sub-tropical or tropical humid forest but not prairies, coastal regions, desert zones or equatorial jungles. The climatic features of the endemic areas is a mild temperature (17-24°C), abundant forest and water resources, short winters and rainy summers (Restrepo, 1985).

A few cases of PCM have been reported outside of Latin America: in the United States, Canada, Europe, Asia and Middle East. With no exception, all the patients had visited one of the countries in the endemic areas. It is interesting that the latency period between the time when the patients departed from the endemic areas and the time when the mycosis became apparent was quite long (mean, 15.3 years). This

observation indicates that *Pb* remains dormant in the human body for years or even tens of years (Brummer et al, 1993).

The ecology of *Pb* is poorly understood. The fungus seems to exist in the environment elusively because it was isolated from soils only occasionally (Brummer et al, 1993). However, it is generally accepted that the habitat of *Pb* is exogenous to humans and people get infected by inhalation of propagules or mycelial fragments from the environment. There have been no reports of outbreaks; nor of person-to-person communication. This might be due to the long latency of the disease which hinders the precise determination of the site of infection. The disease has usually been diagnosed in a place different from where it was acquired. This brings difficulty to precisely locate the source of infection as well as the ecological niche of the fungus. It has been postulated that *Pb* resides in aquatic animals like fish and amphibia in the endemic area (Brummer et al, 1993). Aquatic birds feeding on these reservoirs disseminate the fungus to the environment by excretion. Humans acquire the infection by inhalation of the spores during agricultural activity. However, this hypothesis has not been verified. There is growing evidence that armadillos act as a reservoir. Apart from human beings, armadillos (*Dasypus horemcinctus*) are the only confirmed animals to harbor the fungus. *Pb* strains have been repeatedly isolated from the spleens, livers, and lungs of healthy armadillos (Hebeler-Barbosa et al, 2003), supporting the presumption that the fungus exists in the environment.

In endemic areas, skin tests with paracoccidioidin antigens suggested that substantial amounts of the population have been exposed to the fungus. It is estimated that

about 10 million people have been infected among the 90 million residents in the endemic areas (Brummer et al, 1993). However, only up to 2% of the infected develop overt diseases (McEwen et al, 1995). Even though, the total number of patients with overt disease is substantially large. Indeed, PCM is a major public health threat in Brazil as about 14.6% of hospitalized adults have been reported to have PCM in a local investigation (Ferreira-da-Cruz, 1987). PCM is a fatal disease if not treated. It will become more important in the future with the exploitation of indigenous forest and the development of Brazil.

Skin tests indicate that contact with *Pb* can be as early as in the first 2 decades of life. However, the incidence of the disease is usually low in children and young adults (3-5%). Most cases are found in adults between 30 to 60 years old, and most patients (70%) are connected with agricultural activity or field work (Brummer et al, 1993). The infection has no predilection toward any specific race but indigenous Indians seem to be rarely afflicted whilst immigrants to the endemic area tend to develop severe forms of the mycosis (Brummer et al, 1993). Histocompatibility antigens have been studied in searching the population with relatively high risk for developing PCM. The human leukocyte antigen HLA-B40 seemed to be more often found in patients in Brazil (Lacerda et al, 1988), while HLA-9 and HLA-B13 were thought important in Colombia (Retrepo et al, 1983). However, both investigations were inconclusive.

The PCM has an obvious predilection toward males. The overall rate of the disease in males is 13 times higher than in females (Brummer et al, 1993). This imbalance on gender is not caused by the frequency of exposure because paracoccidioidin skin

tests in healthy individuals from the same area do not reveal any sex difference in subclinical infection. In addition, there is no sex difference in children with overt disease. The reason for the much more frequent progression to the disease in males is that the female hormone β-estradiol plays an important role in preventing females to get an overt infection. *In vitro* experiments confirmed that β-estradiol specifically inhibits the transformation of both mycelia and conidia into the yeast form (Restrepo et al, 1984; Salazar et al, 1988). Meanwhile, an estrogen-binding protein was discovered in the cytosols of *Pb* mycelial and yeast cells (Loose et al, 1983; Stover et al, 1986). The addition of β-estradiol into mycelial cultures before temperature change led to alterations in mycelial protein expression and blocked synthesis of proteins needed for transformation. All these data suggested that β-estradiol functions via specific binding to form a protein-ligand complex in *Pb* cells, thus influencing the fungal behavior and changing the pathogenic consequences. It was hypothesized that if the female host has sufficient levels of estradiols at the time when infectious propagules are inhaled into the body, the propagules will be inhibited from transforming into the yeast form in tissue, giving enough time for the host to mount a specific immune defense and destroy the infecting particles.

1.4 Clinical Manifestations

Paracoccidioidin skin tests in healthy residents in endemic areas revealed that sub-clinical infection occurs quite frequently (Brummer et al, 1993). *Pb* cells have been found in residual, partially calcified lesions in the lungs of healthy people, indicating that fungal growth is halted in competent hosts. It is still unknown how the overt infections start in humans. When conidia get into the lungs, they immediately transform into yeast. These yeast cells could be destroyed by the host; otherwise,

they use their newly synthesized cell wall to avoid host immune surveillance in residual lesions and enter into a resting stage for months or years. There is some evidence that they exist in macrophages like *M. tuberculosis*. It was postulated that transient host-parasite imbalance trigger the progression of the disease (Brummer et al, 1993). The disease starts when the yeast cells change from the dormant to the active state. Yeast cells then use their virulence factors, such as the cell wall component α-glucan, gp43 and melanin, to overcome the host and give rise to the full-blown disease state (Borges-Walmsley et al, 2002).

There are two forms of overt PCM: the acute/sub-acute juvenile form and the chronic adult form (Borges-Walmsley et al, 2002). The acute/sub-acute juvenile form accounts for only 3-5% of all cases (Brummer et al, 1993). It is characterized by a rapid (weeks to months) and severe course. The patients are children and young adults. The clinical manifestation is characterized by hypertrophy of reticuloendothelial system (spleen, liver, lymph nodes, and bone morrow). Lungs are usually not involved and there are no special radiologic manifestations. The hypertrophy of mesenteric lymph nodes can lead to bowel obstruction and an acute abdominal syndrome. The chronic adult form accounts for more than 90% of patients. Most of the patients are between 30 to 60 years old. Unlike the juvenile form, the disease progresses slowly and takes months and years to become fully established. The lung is the organ afflicted, and pulmonary manifestations are usually found in 90% of patients. Symptoms include fever, weight loss, cough, and shortness of breath. X-ray findings are often bilateral and preferentially located in the central and lower portions of the lungs. The lung manifestations resemble those of tuberculosis, with which the mycosis coexists in 10% of cases (Brummer et al,

1993). About 25% of cases are in unifocal form as the lung is the only organ afflicted. The majority of cases are in the multifocal form because extrapulmonary lesions are usually found. Extrapulmonary organs that are most frequently involved are the oral cavity, nasal mucosa, skin, lymph node and adrenal glands (Brummer et al, 1993).

1.5 Immunology

Depressed cell-mediated immunity is a characteristic of PCM patients (Borges-Walmsley et al, 1993). In the acute/sub-acute juvenile form of PCM, cell-mediated immunity is severely depressed in patients, leading to bad prognosis and high mortality (Franco, 1987). In the chronic adult form of the disease, cell-mediated immunity is usually abnormal in the severe, multi-focal form of disease but relatively intact in the less severe uni-focal form. In general, depression of cell-mediated immunity is a common finding in PCM patients and this correlates with the severity of the disease (Mota et al, 1985). The depressed cell-mediated immunity does not seem to be an innate host deficiency but is likely caused by the infectious fungus. There is strong evidence in support of this argument, in that impaired cell immunity is often found to be reversed after successful therapy of the infection (Retrepo et al, 1978).

It was observed that compacted and localized granulomas were indicative of healing of the disease and associated with effective cellular immunity, while diffused granulomas were thought to be correlated with progressive disease and impaired cellular immunity (Borges-Walmsley et al, 2002). The depressed cell-immunity in patients with PCM was represented by reduced CD4/CD8 (helper T-cell/suppressor

T-cell) ratios (Mota et al, 1988), as well as low levels of cytokine production, especially interferon (Borges-Walmsley et al, 2002). DTH (delayed T-cell hypersensitivity) skin testing with paracoccidioidin was used to evaluate the immune status of patients. Depressed reactivity was observed in patients with severe disease of both acute and chronic forms. A reversion from non-reactive to reactive during the course of therapy indicates the restoration of cell-mediated immunity and signals a good prognosis (Brummer et al, 1993). A murine model demonstrated that immunosupression was caused by infection. DTH responses were observed after the first week of infection. However, DTH reactions decreased to insignificant levels by 8 weeks, when antibody titers peaked and disease was severe (Castaneda et al, 1988).

Pb cells can be ingested by monocytes and macrophages and replicate intracellularly (Brummer et al, 1989). However, macrophages activated by γ-interferon have significant ability to kill intracellular *Pb* cells, indicating the important role of interferon in resistance to PCM (Brummer et al, 1988).

In contrast to the depressed cellular immunity, patients with PCM are not deficient in specific antibody production. Almost all the patients with overt clinical manifestations have specific antibodies (Restrepo, 1988; Mendes-Giannini et al, 1990). Levels of antibodies are directly paralleled with the severity of disease. Antibody titers are high in disseminated disease and decreasing antibody titer indicates clinical improvements. The protective role of antibodies in PCM patients has not been determined. Animal models of infection indicated that antibodies may constitute a mechanism of protection (Kamegasawa et al, 1988). However, these studies were inconclusive.

The opportunism of PCM has been studied. Although there is no observation of significant increase of PCM in immunocompromised patients, immunosuppression and immunodeficiency in transplant patients, patients receiving chemotherapy, patients with hemotologic malignancies and AIDS patients are predisposed for activation of latency. Particularly in AIDS patients, the severe acute/sub-acute juvenile form of PCM can develop in adult age, indicating the importance of cell-immunity in control of this mycosis (Brummer et al, 1993).

1.6 Laboratory Diagnosis

The definitive diagnosis of PCM can only be achieved by laboratory procedures (Brummers et al, 1993). The best and fastest way to establish the diagnosis is direct examination of clinical specimens such as sputum or histopathological samples. Multiple budding yeasts like ship-pilot's wheels are the characteristic structure used for diagnosis. Direct examination can identify greater than 85% of PCM patients. Fungal culture is slow, taking at least 20-30 days. The recovery of the fungus from clinical samples is usually made at room temperature (25°C) in order to eliminate contaminating resident flora. Because the mycelial structure is not distinctive, the dimorphic switch must be made by subculture at 37°C to obtain yeast cells. Again, the observation of the pilot's wheel multiple budding yeasts is diagnostic. The isolation rate by fungal culture is greater than 86%.

An agar gel immunodiffusion test was most commonly used in serological diagnosis because it is simple to perform and therefore suitable for small laboratories. About 90% of patients were found to have specific antibodies with this method. It was

specific as well, producing no cross-reactivity with other fungal infections (Retrepo and Moncada, 1974). Research on immunodiagnosis has been emphasized on searching for noval antigen preparations to enhance sensitivity, specificity and reproducibility of tests. Historically, *Pb* antigens have been classified into 3 types: cell-wall antigens, cytoplasmic antigens and culture filtrate antigens. Cell-wall antigens proved to be galactomannans, the usefulness of which was limited because of their cross-reactivity with other fungal infections (Brummer et al, 1993). There were many preparations for cytoplasmic antigens. A typical cytoplasmic antigen is Favo-Netto's polysaccharide antigen, which was prepared from autoclaved yeast cells. This antigen has been widely used as paracoccidioidin in skin test studies (Brummer et al, 1993). Culture filtrate antigens have been frequently used in serodiagnosis of PCM. By using an immunodiffusion test, Restrepo and Moncada (1974) identified 3 precipitation lines, designated 1, 2 and 3. Later, Yarzabal et al (1977) identified 2 antigens E1 and E2 by immunoelectrophoresis. Puccia et al (1986) found a 43 kDa glycoprotein (gp43) as the major specific antigenic component in culture filtrate. The gp43 proved to be Restrepo and Moncada's line 1 and Yarzabal's E2 antigen. It is continuously excreted into the culture medium by yeast cells during the exponential phase of growth (Stambuk et al, 1998). When used in Western blot, gp43 was recognized by 100% of patients' sera (Camargo et al, 1989). The deglycosylated product of gp43 has a molecular weight of 38 kDa (Puccia and Travassos, 1991a). Peptide epitopes of gp43 were mapped and they were immunodominant in their reactions with patient and animal sera. Cross-reactivities of gp43 with other fungal infections were attributed to carbohydrate epitopes but not the protein part (Puccia and Travassos, 1991b). The gp43 is not only excreted into the culture medium but also released into the blood stream of

patients. Antigenemia of gp43 has been detected in 100% of patient sera (Mendes-Giannini et al, 1989; Marques da Silver et al, 2003 and 2004). The gp43 has prognostic value as well because it was detected in patients without treatment but its concentration in patient sera decreased during the course of treatment. Concurrently, the decreased antigenemia was accompanied by a reduction in anti-gp43 antibodies in patient sera. Therefore, gp43, especially the recombinant protein portion, will be an excellent candidate antigen with high sensitivity, specificity and reproducibility in the application of diagnosis and monitoring the efficacy of treatment.

1.7 Therapy

Paracoccidioidomycosis used to be an incurable disease. It can be treated currently but the remission takes a long time (at least half a year; Brummer et al, 1993). Relapse is still a problem that needs to be solved by novel antifungal drugs. Sulfonamides and amphotericin B used to be the drugs for treatment. However, they are not chosen in clinical use today because of shortcomings, such as the long term of treatment, severe side effects, difficulty in administration and high rate of relapse (up to 30%). The current drugs of choice are ketoconazole and itroconazole. When ketoconazole is administered at a dose of 200-400 mg/day for 12 months or less, about 95% of patients are responsive, and the relapse rate is about 11% at 3 years after therapy. Itraconazole seems superior to ketocanazole. The daily dose of itraconazole is lower (100 mg) and the period of therapy is shorter (6 months). The response rate is the same as ketoconazole but with a lower relapse rate (3-5%). Although the disease can be treated, recovery is often accompanied by fibrotic sequelae in the lungs, which are a cause of pain for the whole life of the patients (Brummer et al, 1993; Borges-Walmsley et al, 2002).

1.8 Initiation of *P. brasiliensis* Studies at the Gene Level

As reviewed above, the basic pathobiology of *P. brasiliensis*, the disease paracoddidiodomycosis (PCM) and its immunology, have been characterized mainly by scientists in South America where the disease is endemic. However, further understanding of the biology of this fascinating fungus requires research at the gene level. At the beginning of our research project in 2000, only limited number of papers had been published concerning the molecular biology of this fungus (da Silva et al, 1999). In deed, at that time the number of genes of *Pb* available in the GenBank was only a dozen. The progress of research was severely hindered by lack of gene information, molecular genetic tools, such as vectors for transformation and over-expression of individual genes, and gene knock-out techniques. Forward and reverse genetics could not be applied and therefore it was difficult to investigate the role of individual genes involved in the virulence and the progression of the disease.

The most significant characteristic of *Pb* is its dimorphic switch, which correlates with its virulence since mutants unable to switch forms are less virulent (Borges-Walmsley et al, 2002). Interestingly, temperature change is the only factor that is needed to cause this switch. However, there is little information in how the organism senses and responds to the temperature change (da Silva et al, 1999). We chose to study the cell signaling pathways, especially the cAMP signaling pathway, as the entry point for the molecular biology of *Pb*. The reason is as follows: as early as in 1985, it was demonstrated that exogenous cAMP (either added in the medium or induced by inhibitors of phosphodieaterase) inhibits the yeast to mycelial transition in *Pb* and elevated levels of cAMP were supposed to support the transition to the yeast form of *Pb* (Paris and Duran, 1985). This indicated that the dimorphic change

is under the control of the cAMP signaling pathway. For several other dimorphic pathogenic fungi, changes in cAMP levels have been found to correlate with morphological changes. The yeast *Candida albicans* can be induced to undergo germ tube or mycelia formation under a number of culture conditions and in host tissues. Changes in temperature seem to be critical; germ tube formation induced at 40°C in a defined medium was accompanied by an increase of cAMP concentration in the cell; and exogenous cAMP, either added to the medium as dibytyryl cAMP or induced by cAMP phosphodiesterase inhibitors, induced germ tube formation at a lower temperature (32°C or 25°C; Niimi et al, 1980; Sabie and Gadd, 1992). *Histoplasma capsulatum*, a pathogenic dimorphic fungus causing the human disease histoplasmosis, grows as mycelia at 25°C in soil and as yeast at 37°C in tissues. During the temperature-induced transition from yeast to mycelia, there was a 5-fold increase in intracellular cAMP levels and more than 15-fold accumulation of cAMP in the culture medium (Medoff et al, 1981). Exogenous cAMP induced mycelium formation at the nonpermissive temperature of 37°C (Medoff et al, 1981). The dimorphic fungus *Mucor racemoses* was found to have an increased intracellular cAMP level during the morphologic change from an air-grown mycelium form to an anaerobic yeast form. The addition of dibutyryl cAMP to mycelia under aerobic conditions resulted in yeast like development (Paznokas and Sypherd, 1975).

Interestingly, the model yeast *S. cerevisiae* has also been observed to have a morphological change from vegetative to pseudohyphal (or filamentous) growth when starved of nitrogen in the presence of a fermentable carbon source (Gimeno et al, 1992). This morphological change has been shown to be controlled by at least two cell signaling pathways, i.e. the MAP kinase pathway and the cAMP signaling

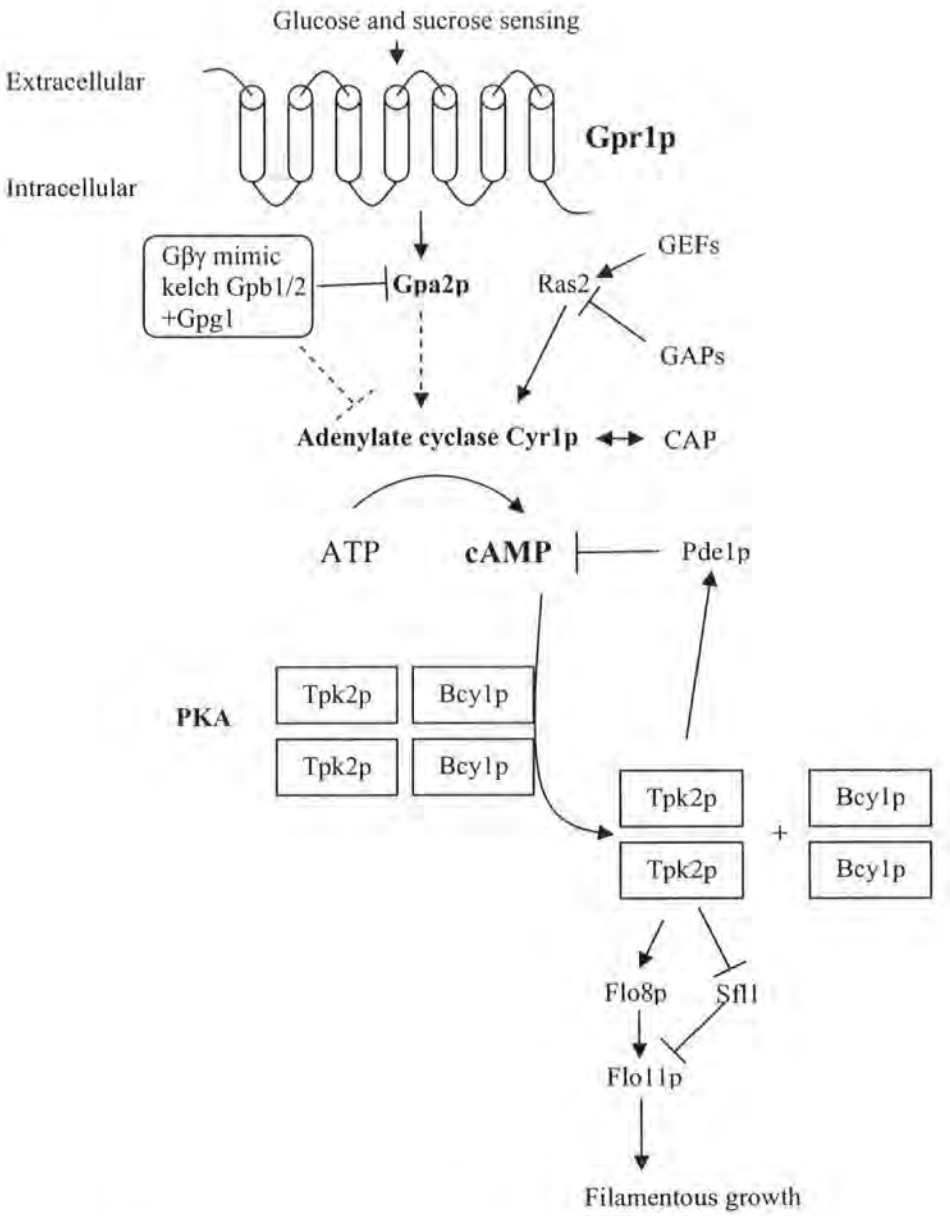
pathway (Lengeler et al, 2000). In fact, most of our knowledge of the cell signaling pathways controlling morphological switching in pathogenic fungi came from the research on pseudohyphal growth of *S. cerevisiae* (Borges-Walmsley and Walmsley, 2000). Current research indicates that pathogenic fungi use similar components and mechanism in controlling the dimorphic switch between saprobic and pathogenic forms. Given that morphological changes in dimorphic fungal pathogens often underlie the development of pathogenicity (Lo et al, 1997; Wickes et al, 1996), studies of the genes, such as those in the cAMP signaling pathway that control the morphological change, will facilitate discovery of new targets for antifungal drugs. With this objective in mind, we initiated a study of the cAMP signaling pathway in *P. brasilinesis* by cloning the major components of the pathway and analyzing their structure-function relationship. In the following part of this chapter, I will review in detail the current understanding of the signaling pathways that control the pseudohyphal growth in *S. cerevisiae* with emphasis on the cAMP pathway. The pheromone mating pathway of the yeast *S. cerevisiae* is also reviewed as this will help in understanding G-protein regulation. In addition, signaling controls of dimorphic switching or virulence in some important pathogenic fungi are given as well.

1.9 The G protein-cAMP-PKA Signalling Pathway of the Yeast *S. cerevisiae*: Glucose Sensing and Pseudohyphal Growth

1.9.1 Adenylate Cyclase *CYR1* and Cyclase-Associated Protein *CAP*

A central component of the cAMP signaling pathway is adenylate cyclase (Figure 1.9; adapted from Borges-Walmsley and Walmsley, 2000), which catalyzes the synthesis of cAMP from ATP. The adenylate cyclase gene is essential as yeast cells

Figure 1.9. Summary of the cAMP-signaling pathway in *S. cerevisiae*



Bcy1p: protein kinase A regulatory subunit
GEFs: guanine nucleotide exchange factors
GAPs: GTPase activating proteins
Pde: phosphodiesterase
PKA: protein kinase A
Tpk: protein kinase A catalytic subunit

are non-viable when the gene is deleted (Borges-Walmsley and Walmsley, 2000). The interests in yeast adenylate cyclase started following the observation of a change in cAMP levels during the dimorphic switch in some pathogenic fungi. By isolating yeast mutants that require cAMP for growth, the adenylate cyclase gene of *S. cerevisiae* was first mapped at a chromosome locus, termed *cyr1* (*cy*clic AMP *r*equirement), located near the centromere of chromosome X (Matsumoto et al, 1982). The *CYR1* gene was cloned by complementation of a *cyr1* strain deficient in adenylate cyclase activity with a yeast genomic DNA library (Kataoka et al, 1985). The DNA sequence of *CYR1* encodes a protein of 2,026 amino acids. When analyzed by SMART (EMBL) today, the Cyr1 protein contains at least four domains, i.e. a RA (Ras association; 676-756) domain, 11 LRR (23 amino acid Leucine-Rich Repeats; 885-1233) domains, a PP2Cc domain (1347-1622) and a catalytic domain CYCc (1611-1833). The isolated catalytic domain retains a Mn^{2+}-dependent catalytic activity, while the remaining portion of the protein was proposed to be essential for the GTP-dependent activation by RAS protein in the presence of Mg^{2+} (Kataoka et al, 1985). In yeast, adenylate cyclase activity was found in the insoluble fraction of cells extracted without detergent, indicating that the yeast adenylate cyclase is membrane-associated (Kataoka et al, 1985). However, the yeast adenylate cyclase is not an integral membrane protein because no membrane-spanning domains have been found. That adenylate cyclase is only peripherally associated with membrane is based on the following facts: adenylate cyclase activity was found in both membrane and cytoplasmic fractions; adenylate cyclase activity could be released from membrane, simply by the addition of 0.5 M NaCl; activity from both cytosolic fractions and NaCl extracts could be functionally reconstituted into membranes lacking adenylate cyclase activity (Mitts et al, 1990). The amphipathic

LRR domains were supposed to be responsible for the membrane localization because deletion of the LRR domains redirected the adynelate cyclase activity from the membrane portion to the cytosolic portion (Mitts et al, 1990). In another experiment, the adenylate cycalse activity located predominantly in the high speed pellet fraction when adenylate cyclase containing the LRR repeats was over-expressed in *E. coli*, while the acenylate cyclase activity located predominantly in the high speed supernatant fraction when the LRR repeats were deleted (Kataoka et al 1985). These results indicated that the adenylate cyclase may be anchored to the membrane by interacting with a membrane-associated protein. Interestingly, adenylyl cyclase activity in *ras1ras2byc1* cells was found in the soluble fraction, while it was found in the membrane fraction in the wild-type strain. Overexpression of the gene *CDC25*, encoding a guanine exchange factor for RAS proteins, in the *ras1ras2bcy1* strain relocalized adenylyl cyclase activity to the membrane fraction (Engelberg et al, 1990). In addition, deletion of *IRA1*, the gene encoding a GTPase-activating protein for RAS proteins, dislocated the adenylate cyclase activity from the membrane (Mitts et al, 1991). Both Cdc25 and Ira1 could complex with adenylate cyclase. These findings suggested that the membrane localization of adenylate cyclase is correlated with the RAS proteins, Cdc25 and Ira1 through the formation of a large complex.

In fact, yeast adenylate cyclase exists as two enzyme complexes that associate with the membrane when fractionated *in vitro* (Wang et al, 1992). One complex, with a molecular weight of 890 kDa, at least consists of adenylate cyclase and another 70-kDa protein component, termed CAP (cyclase-associated protein). The adenylate cyclase activity in this complex is Ras-dependent in the presence of Mg^{2+}. Another

cyclase containing complex of 670 kDa was CAP free. Its adenylate cyclase activity is Mn^{2+}-dependent. Missense mutations in the leucine-rich repeats abolished the Ras-dependent acenylate cyclase activity and changed it into Mn^{2+}-dependent (Wang et al, 1992). In another report, adenylate cyclase and CAP were co-purified, indicating that they are tightly associated (Field et al, 1988). The gene encoding CAP was cloned by screening a yeast cDNA expression library with antisera against the 70-kDa component. Adenylate cyclase in the yeast cell membrane lacking CAP was not stimulated by Ras2 *in vitro*, indicating that CAP is essential for Ras activation of adenylate cyclase (Field et al, 1990a). The CAP-binding site of adenylate cyclase was mapped to the last 148 residues at the C-terminal end. Overexpression of this small segment of adenylate cyclase sequestered the CAP protein and suppressed the heat shock sensitivity of yeast cells bearing the dominant active RAS^{G19V} allele (Wang et al, 1993). CAP is necessary for the stimulatory effect of post-translationally modified Ras, but not for the unmodified Ras, and the region of CAP required for this effect was mapped to its N-terminal 168 residues (Shima et al, 1997). The N-terminus of CAP was proposed to bind to the C-terminus of adenylate cyclase through a coiled-coil interaction (Shima et al, 2000).

CAP is a bifunctional protein. Its primary structure contains N-terminal and C-terminal domains that are separated by a proline-rich region. Its N-terminal 36 residues bind to a segment of adenylate cyclase (1898-2016) near the C-terminal end of adenylate cyclase (demonstrated by yeast two-hybrid assay) in a coiled-coil interaction, and this binding mediates the activation of adenylate cyclase by RAS proteins (Nishida et al, 1988). The C-terminal domain of CAP is necessary for normal cellular morphology (cytoskeleton phenotype) and responses to nutrient

extremes (Vojtek et al, 1991). It has been demonstrated (by yeast two-hybrid assay and immunoprecipitation) that the C-terminal half of CAP binds both actin monomers and the actin polymer directly (Freeman et al, 1995; Zelicof et al, 1996). In addition, CAP forms a dimer via its last 27 amino acid residues (Zelicof et al, 1996). The middle proline-rich region of CAP interacts with the SH3 (Src homology 3) domian of Abp1, a yeast actin-binding protein which in turn binds CAP to cortical actin (Freeman et al, 1996). These data suggest that CAP and adenylate cycalse are localized with actin.

Although yeast adenylate cyclase appears to be central to the cAMP signaling pathway, it is difficult to demonstrate directly because the gene is essential for the cell viability and its deletion is lethal. However, a mutation near the end of the adenylate cyclase catalytic domain, $Cyr1^{K1876M}$, was isolated and demonstrated to be able to maintain a basal cAMP level but failed to mount a transient increase in cAMP in response to glucose or intracellular acidification, confirming the key role of adenylate cyclase in transducing the cAMP signal (Vanhalewyn et al, 1999).

1.9.2 Phenotypes of Pathway Activation

The yeast *S. cerevisiae* cAMP signaling pathway was first demonstrated to be associated with glucose sensing, and later found to be correlated with pseudohyphal growth. There are two types of stimulation that trigger the activation of the cAMP signaling pathway (Mbonyi et al, 1988). First, addition of glucose or related fermentable carbon sources to the yeast cells grown on non-fermentable carbon sources (so-called derepressed cells) induces a transient peak in the cAMP signal. The activation of the pathway leads to the rapid depletion of the storage sugar

trehalose, inhibition of gluconeogenesis and stimulation of glycolysis. Cells grown on fermentable sugars have a higher basal cAMP level than cells grown on non-fermentable carbon sources (e.g. ethanol, glycerol and acetate). Second, drastic lowering of the intracellular pH (e.g. by addition of dinitrophenol) in yeast cells strongly stimulates *in vivo* cAMP synthesis. The characteristics for activated cAMP signaling pathway are: an enhanced growth rate, depletion of storage carbohydrates such as glycogen, sensitivity to heat shock (growth defect at high temperature) and starvation, and defective in sporulation of diploid cells. On the other hand, reduced cAMP signaling causes increased accumulation of storage carbohydrate and sporulation even in rich medium and entry into stationary phase (Kataoka et al, 1984).

The glucose-induced and intracellular acidification-induced activation of the cAMP pathway are mediated by RAS proteins because the cAMP signal is absent in the *RAS1-RAS2-bcy1* double deletion strains (*RAS* deficiency is suppressed by the *bcy1* mutation; Mbonyi et al, 1988; Colombo et al, 1998). In addition, the dominant active $RAS2^{G19V}$ mutation failed to mediate glucose or intracellular acidification by mounting a transient peak of cAMP in a strain with repressed *RAS1*, although it can constitutively activate the adenylate cyclase to produce a higher cAMP level *in vivo*. In contrast, either *RAS1* or *RAS2* alone can induce a prominent transient cAMP peak. Glucose induced Ras activation is dependent on glucose transportation and intracellular phosphorylation and the process of activation is probably through inhibition of the Ras-GAPs (Ras GTPase-activating protein) Ira1/2 (Colombo et al, 2004).

Glucose-induced but not intracellular acidification-induced cAMP signaling is also dependent on the G protein α subunit gene *GPA2*. Overexpression of *GPA2* enhanced glucose-induced cAMP levels and suppressed the growth defect of a temperature-sensitive *ras2* mutant (Nakafuku et al, 1988). Constitutive $GPA2^{R273A}$ conferred heat shock sensitivity and a sporulation defect, indicative of activation of the cAMP pathway (Xue et al, 1998). In contrast, the glucose-induced increase in cAMP was absent in Δ*gpa2* strains (Colombo et al, 1998). Indeed, Gpa2 is coupled to Gpr1, the membrane receptor whose agonist is glucose (Lemaire et al, 2004). Glucose activation of Gpr1 does not need phosphorylation.

The cAMP signaling pathway also regulates yeast pseudohyphal differentiation. In the diploid yeast, nitrogen starvation in the presence of carbon sources induces pseudohyphal or filamentous development (Gimeno et al, 1992). The pseudohyphal cells are elongated in shape and are attached to each other by their cell wall. The cells undergo a unipolar budding pattern in which daughter cells bud at the pole opposite the mother cells. The result is a filamentous, branched display of cells growing away from the centre of a colony and invading the agar medium (Borges-Walmsley and Walmsley, 2000; Pan et al, 2000). Therefore, there are four phenotypes that are distinct and highly correlated in the process of filamentation: uni-polar budding, cell elongation, incomplete cell separation and invasive growth. The filamentous growth may allow this non-motile fungus to forage for nutrient in an adverse environment (Borges-Walmsley and Walmsley, 2000). Haploid cells have the axial budding pattern that precludes pseudohyphal growth when starved for nitrogen. However, haploid cells can invade the agar when grown in rich medium (Borges-Walmsley and Walmsley, 2000). The genes required for diploid

pseudohyphal growth are also necessary for haploid invasive growth (Lengeler et al, 2000). Therefore, in this thesis, when pseudohyphal growth is mentioned, only the diploid situation is referred to unless otherwise stated.

Because filamentous growth is induced by nitrogen starvation, nitrogen sources such as ammonium or amino acids may act as stimulants for pathway activation. The expression of the membrane receptor Gpr1 and the Tpk2 (catalytic subunit of PKA) are up-regulated upon nitrogen starvation (Xue et al, 1998; Pan and Heitman, 1999). A high affinity ammonium permease Mep2 was found to be required for pseudohyphal development upon ammonium limitation (Lorenz and Heitman, 1998a). The pseudohyphal defect of *Δmep2/Δmep2* can be suppressed by dominant active Ras2, Gpa2 and exogenous cAMP; but not by activated alleles of the MAPK pathway, indicating that Mep2 might be a component in the upstream cAMP pathway. Mep2 probably lost its original role as an ammonium transporter during evolution because *Δmep2/Δmep2* mutants have no obvious change in ammonium uptake. A possible role for Mep2 is as an ammonium starvation sensor, which regulates filamentation. In addition, another ammonium permease Mep1, with low ammonium affinity, is an inhibitor of pseudohyphal growth and is correlated with the MAPK pathway (Lorenz and Heitman, 1998b).

Filamentous growth is partly regulated by the cAMP signaling pathway; but as discussed later, it is also regulated by MAPK pathway. The dominant active $RAS2^{G19V}$ gene stimulates filamentous growth, apparently by increasing the cAMP level in the cells (Gimeno et al, 1992). However, it also induces filamentous growth by activating the MAPK pathway (Mosch et al, 1996). Lowering cAMP level in

RAS2^{G19V} cells by overexpression of the phophodiesterase Pde2 suppresses filamentous growth, indicative of a correlation of cAMP level and filamentous growth (Ward et al, 1995). In fact, exogenous cAMP in the media (10 mM) stimulates filamentous growth in wild-type strains even in the absence of nitrogen starvation (Lorenz and Heitman, 1997). The filamentous growth is highly correlated to glucose stimulation, firstly because morphogenesis occurs in the presence of glucose and absence of nitrogen; and, secondly, the Gpa2 coupled membrane receptor Gpr1 is a glucose sensor (Lemaire et al, 2004). The details of the cAMP signalling pathway and its role in filamentous growth are discussed below.

1.9.3 RAS Proteins

The first regulator of adenylate cyclase was found to be RAS proteins (Toda et al, 1985; Broek et al, 1985). In mammalian cells, RAS proteins are small guanine-nucleotide-binding proteins that possess intrinsic GTPase activity. Ras cycles between an active GTP-bound form and an inactive GDP-bound form. The yeast has two *RAS* genes, *RAS1* and *RAS2*. Genetic analysis of *RAS1* and *RAS2* indicated that neither *RAS1* nor *RAS2* are essential individually but *ras1-ras2-* double deletion mutants are not viable. Yeast RAS proteins are structurally similar to their mammalian counterparts particularly in their N-terminal half. Yeast RAS proteins are membrane associated. At the C-terminus of the RAS proteins, there are conserved CAAX motifs (C, cysteine; A, aliphatic; and X, any amino acid) that were responsible for palmitoylation or farnesylation of the cysteine residue (Kuroda et al, 1993). Both yeast and mammalian RAS proteins undergo similar processing events and localize to membrane fractions. In yeast, palmitoylation is responsible for membrane localization, while farnesylation is responsible for the activation of

adenylate cyclase. In one study, farnesylated Ras2 had 100 times higher activity in stimulation of adenylate cyclase than the unprocessed form (Kuroda et al, 1993). In another study, although farnesylation of Ras proteins had no significant effect on the cyclase binding, it was shown to increase Ras activity 5- to 10-fold and this effect was dependent on the N-terminus of the cyclase-associated protein CAP (Shima et al, 1997).

Twenty years ago, RAS proteins were proposed to be the direct activator of adynelate cyclase. The catalytic domain of adenylate cyclase alone can produce cAMP irrespective of the presence of RAS proteins. However, adenylate cyclase produces increased amounts of cAMP in the presence of RAS proteins (Uno et al, 1987; Toda et al, 1985). It is generally accepted that the Ras proteins are in the GTP-bound state when activated and turned off by GTP hydrolysis. A dominant active mutation $RAS2^{G19V}$ activates adenylate cyclase in a GTP-independent manner. The mutation point is the second Gly in the GXGXXG motif (where the X is any amino acid). $RAS2^{G19V}$ encodes a protein that has reduced GTPase activity and constitutively activates the *CYR1* gene product. Diploid yeast cells containing this mutation are sensitive to heat shock and starvation, and are incapable of sporulating efficiently (Kataoka et al, 1984). The RAS2 protein can not activate adenylate cyclase when bound to GDP or a stable GDP analog GDP-βS. In a study, when purified from *E. coli,* the RAS2 protein did not have GTP bound, and therefore was not able to activate adenylate cyclase *in vitro* (Field et al, 1988). Membranes from a *ras1-ras2-bcy1* strain lack GTP-stimulated adenylate cyclase activity. Mixing membranes of a *ras1-ras2-* yeast strain with membranes from an adenylate cyclase deficient yeast strain reconstituted GTP-dependent adenylate cyclase activity (Toda

et al, 1985). In addition, a direct relationship between Ras2 and Cyr1 was confirmed by reconstitution in an *E. coli* system both *in vivo* and *in vitro* (Uno et al, 1985). *E. coli* transformant cells carrying both *CYR1* and *RAS2* (or the active dominant mutation $RAS2^{G19V}$) produced GTP-dependent (GTP-independent for $RAS2^{G19V}$) adenylate cyclase activity and cAMP, while *E. coli* cells carrying *CYR1* or *RAS2* alone had no measurable cAMP. Mixing the crude extract of *E. coli* carrying *CYR1* with the extract of *E. coli* carrying *RAS2* reconstituted the GTP-dependent adenylate cyclase activity.

RAS proteins can directly bind to adenylate cyclase in both a modified and unmodified form as indicated by immunoprecipitation of purified adenylate cyclase and RAS proteins, and this interaction is dependent on GTP but independent of CAP (Shima et al, 1997). Deletion and insertion mutations in the adenylate cyclase gene were used to map domains required for activation by RAS proteins. In one research, it was demonstrated that the 0.8 kb region adjacent to that encoding the catalytic domain (e.g. the PP2Cc domain) is associated with the regulatory function of RAS proteins (Uno et al, 1987). From other research, the N-terminal 605 amino acids were found to be dispensable for responsiveness to Ras, while the LRR domains (which were mapped to 674-1301) were found to be essential for Ras activation (Colicelli et al, 1990; Field et al, 1990; Suzuki et al, 1990). Interestingly, in *Trypanosoma equiperdum*, an open reading frame encoding a protein sequence similar to the leucine-rich repeats in *S. serevesiae* was found adjacent to its adenylate cyclase, suggesting that the two proteins may interact to regulate adenylate cyclase activity (Ross et al, 1991). Recently, an RA (Ras association) domain (676-756) was found in the previously so-called LRR domains (Kido et al, 2002). The purified RA

domain polypeptide directly binds to Ras in a GTP-dependent manner as demonstrated by immunoprecipitation. This domain competitively inhibits Ras-dependent activation of adenylate cyclase both *in vivo* and *in vitro*. RA is the primary binding site for RAS proteins.

Although farnesylation of Ras and CAP are not essential for Ras interaction with adenylate cyclase, they are crucial for its activation (Shima et al, 2000). Immunoprecipitation experiments demonstrated that the complex of CAP and the C-terminal-region of Cyr1 (1764-2026) form a second Ras-binding site which binds modified Ras only. This is consistent with the fact that the last 66 C-terminal residues of adenylate cyclase are indispensable for Ras activation (Suzuki et al, 1990). However, neither CAP nor Cyr1 (1764-2026) alone can bind modified Ras. This second Ras-binding site is mainly used for adenylate cyclase activation because CAP mutants that increase or decrease Ras-binding at this site lead to deficiency in adenylate cyclase activation (Shima et al, 2000).

Although both Ras1 and Ras2 activate the cAMP signaling pathway, Ras2 is a much more potent activator than Ras1. The cAMP level in *ras2-* cells is lower than in *ras1-* cells (Toda et al, 1985), and Ras2 acitvates adenylate cyclase more efficiently than Ras1 (Broek et al, 1985). The difference in efficiency of adenylate cyclase activation between Ras1 and Ras2 resides in the highly conserved N-terminal domain rather than the variable C-terminal domain as indicated by chimeric constructions of *RAS* genes by swapping the sequences encoding the variable C-terminal domains (Hurwitz et al, 1995).

The function of yeast Ras is regulated by the GEFs (guanine exchange factors) Cdc25 and Sdc25, which catalyze GTP exchange, activating Ras; and by the GAPs (GTPase-activating proteins) Ira1 and Ira2, which accelerate the intrinsic GTPase activity of Ras and inactivate it (Borges- Walmsley and Walmsley, 2000). Defective mutations in *ira1* and *ira2* leads to constitutive activation of the cAMP pathway, while disruption of *CDC25* causes deficiency in glucose-induced increase in cAMP production (van Aelst et al, 1991). Cdc25 is a 180 kDa membrane bound protein that is essential for cAMP production (Garreau et al, 1990). It is under the direct positive control of Ssa1, which is a chaperone of the HSP70 superfamily (Geymonat et al, 1998). Deletion of Ssa1 leads to reduced content of Cdc25 in the cell and reduced activity of the cAMP-dependent PKA pathway.

As mentioned above, RAS proteins mediate glucose signaling. Another important function for RAS proteins is to regulate filamentous growth. The dominant active mutation $RAS2^{G19V}$ stimulates pseudohyhal differentiation through an increasing cAMP level (Gimeno et al, 1992; Ward, 1995). Deletion of *RAS2* results in reduced filamentation, probably due to a decreased cAMP level in the cell, while the dominant active $RAS2^{G19V}$ allele enhances filamentous growth (Lorenz and Heitman, 1997). In addition, Ras2 is also an activator of the MAPK pathway which will be reviewed in the later part of this chapter.

1.9.4 *GPA2*, *GPB1,2*, and *GPG1*

Yeast *GPA2* was cloned by low stringency hybridization with a rat G protein α subunit. *GPA2* is homologous to mammalian G protein α subunits except for a stretch of 83 additional amino acid residues near the N terminus. Over-expression of

Gpa2 in yeast restored glucose-induced cAMP formation in a *ras2* mutant, indicating that Gpa2 regulates the level of cAMP in addition to RAS proteins (Nakafuku et al, 1988). The major function of Gpa2 is to positively regulate filamentous or pseudohyphal growth in yeast. A *Δgpa2/Δgpa2* mutant strain has a defect in pseudohyphal growth that can be rescued by exogenous cAMP in the media (a *Δras2/Δras2* strain can still form filaments, indicating that Gpa2 is more potent than Ras2 in regulating filamentation; Kubler et al, 1997; Lorenz and Heitman, 1997). In addition, a dominant negative $GPA2^{G299A}$ allele (supposed to prevent a conformational change necessary for GTP binding) inhibits filamentation of wild-type strains. In contrast, a dominant active allele $GPA2^{G132V}$ or $GPA2^{Q300L}$ (with decreased GTPase activity) stimulate filamentation even on nitrogen-rich media (Lorenz and Heitman, 1997; Harashima and Heitman, 2002).

GPA2 is upstream of adenylate cyclase in the pathway because exogenous cAMP suppresses the *Δgpa2* pseudohyphal defect (Lorenz and Heitman, 1997). It is proposed that Gpa2 directly activates adenylate cyclase. To date, however, there is not direct evidence to prove this hypothesis. Unlike RAS proteins, Gpa2 does not regulate the MAPK pathway. Neither dominant active *STE11-4* (Mosch et al, 1996) nor overexpression of *STE12* suppresses the defect of filamentation conferred by a *Δgpa2/Δgpa2* mutation. None of the deletions of the MAPK pathway components *STE20*, *STE11*, *STE7* and *STE12* blocks the dominant active $GPA2^{G132V}$ to simulate pseudohyphal growth. Overexpression of the dominant active $GPA2^{G132V}$ does not activate the MAPK pathway activation reporter *FG(TyA)::lacZ* (Lorenz and Heitman, 1997).

Gpa2 is negatively regulated by Rgs2, a member of regulators of heterotrimeric G protein signaling (Versele et al, 1999). Rgs2 can directly interact with Gpa2, but preferably with Gpa2 in the GTP-bound state, as demonstrated by yeast two-hybrid assays and immunoprecipitation. Rgs2 accelerates the hydrolysis of GTP on Gpa2. The dominant active $GPA2^{G132V}$ allele abolishes the effects of Rgs2 in terms of both the cAMP level and the phenotypes of cAMP pathway activation (Versele et al, 1999). Gpa2 is coupled to the upstream membrane receptor Gpr1, whose major role is glucose sensing (Kraakman et al, 1999).

Canonical G proteins exist as heterotrimers comprised of α, β and γ subunits. Signaling through the conformational change in the G protein-coupled receptor promotes GDP-GTP exchange on the G protein α subunit, and releases the Gβγ complex. In mammalian cells, both Gα and Gβγ can induce downstream signaling events (Hoffman, 2005a). However, the yeast Gα protein Gpa2 lacks the traditional βγ subunits (Lorenz and Heitman, 1997). The pheromone-responsive βγ complex (Ste4 and Ste18) is not involved in pheudohyphal growth and thus has no relationship with Gpa2. There are another 8 candidate β genes and 3 candidate γ genes in the yeast genome. However, none of these genes has a role in pseudohyphal differentiation (Lorenz and Heitman, 1997). The question is whether Gpa2 functions alone or in concert with other partners. Recently, it has been demonstrated that Gpa2 binds to kelch repeat proteins Gpb2/Krh1 and Gpb1/Krh2, which serve as Gβ subunit mimics, and Gpg1 which serves as Gγ mimic (Harashima and Heitman, 2002; Batlle et al, 2003; Harashima and Heitman, 2005). The Gβγ mimic negatively regulates Gpa2 and also negatively regulates its own effector that is unidentified, but could possibly be adenylate cyclase.

GPB2/KRH1, *GPB1/KRH2* and *GPG1* were identified by yeast two-hybrid screening with *GPA2* (Harashima and Heitman, 2002; Batlle et al, 2003). The Gpa2-Gpg1 interaction is indirect and mediated by Gpb1 or Gpb2 because the interaction is diminished in a strain with both *gpb1* and *gpb2* deleted. Gpg1 interacts with both Gpb1 and Gpb2. Sequence analysis indicates that Gpb1 and Gpb2 both contain seven kelch repeats and they are homologous to several kelch repeat proteins (Harashima and Heitman, 2002). The kelch repeat segment is about 50 amino-acid long and is characterized by a double glycine motif following four hydrophobic residues (Adams et al, 2000). Kelch repeats have been implicated in protein-protein interactions. The crystal structure of one kelch repeat protein indicates that these proteins are folded into a seven-bladed propeller structure that is strikingly similar to the structure of G protein β subunits (Ito et al, 1994). Gpg1 is a small protein containing only 126 amino acid residues. It is similar in size to known Gγ subunits and its secondary structure is predicted to be two linked α-helical domains, similar to known Gγ subunits as well. However, Gpg1 has no C-terminal motifs for posttranslational modifications such as farnesylation, myristoylation or palmitoylation. In general, Gpb1/2 and Gpg1 form an atypical complex, which mimics the Gβγ subunits, and interacts with Gpa2. Gpb1/2 interacts with both the GDP and GTP-charged forms of Gpa2, but preferentially with the GDP-charged form: as demonstrated by both yeast two-hybrid assays and immunoprecipitation (Harashima and Heitman, 2002). However, another group reported that only the C-terminus of Gpb2/Krh1 interacts with Gpa2, and preferentially with the dominant active $Gpa2^{R237A}$ (Batlle et al, 2003).

Genetic analyses indicate that Gpb1/2 negatively regulates the cAMP signalling pathway by both Gpa2-dependent and Gpa2–independent mechanisms (Harashima and Heitman, 2002). A *Δgpb1Δgpb2* mutant exhibits phenotypes of the activated cAMP pathway, including hyperfilamentous growth, increased sensitivity to heat shock, reduced glycogen storage and reduced sporulation. These phenotypes were fully abolished by disruption of *TPK2*. Interestingly, introduction of *Δgpa2* to the *Δgpb1Δgpb2* mutant only partially reduces the hyperfilamentous growth. Furthermore, the expression of Flo11 in *Δgpa2Δgpb1Δgpb2* cell is two- to three-fold lower than that in *Δgpb1Δgpb2* cells, but higher than in *Δgpa2* cells (Harashima and Heitman, 2002). These results indicate that Gpb1 and Gpb2 must have another target in addition to Gpa2. This target is upstream of and might include Tpk2 itself (Lu and Hirsch, 2005). Adenylate cyclase is one of the candidates but to date there is no direct evidence to prove this (Ivey and Hoffman, 2002). Kelch repeat proteins can bind different proteins simultaneously on opposing surfaces of the β propeller structure (Adams et al, 2000). Therefore, the Gβγ mimic Gpb1/2-Gpg1 could bind both Gpa2 and adenylate cyclase but using different domains.

Gpa2 in both the GDP and GTP-binding state can interact with its upstream membrane receptor, Gpr1, but the interaction is stronger when it is in the GDP-binding state (Harashima and Heitman, 2002). In addition, the Gpa2-Gpr1 interaction is independent of Gpb1 and Gpb2, because Gpa2 is still functional in a *Δgpb1Δgpb2* strain. However, the transient peak of cAMP production upon glucose stimulation exists in either single *Δgpb1* or single *Δgpb2* mutants, but diminishes in a double *Δgpb1Δgpb2* mutant strain, indicating that Gpb1/2 modulates Gpa2-Gpr1 coupling (Harashima and Heitman, 2002). It was demonstrated that Gpb1/2 inhibits

Gpa2-Gpr1 coupling (Harashima and Heitman, 2005). This is in contrast to canonical Gβ subunits which promote interactions of the Gα-GPCR interaction.

1.9.5 *GPR1* and *PLC1*

The Gpa2 coupled receptor Gpr1 was cloned by yeast two-hybrid screening with Gpa2. The Gpr1 is a typical membrane receptor with 7 transmembrane domains. It interacts with Gpa2 with its C-terminal cytoplasmic tail (Yun et al, 1997; Xue et al, 1998). Although the interaction is demonstrated to be direct in the yeast two-hybrid system, it does not necessarily reflect the situation *in vivo*. In another study, the interaction between Gpr1 and Gpa2 was reported to be mediated by Plc1, a phosphatidylinositol-specific phospolipase C, which hydrolyzes the membrane phospholipids phosphatidylinositol 4,5-biphosphate (PIP2) to produce the second messengers inositol 1,4,5-triphosphate and diacylglycerol (Ansari et al, 1999). Co-immunoprecipitation experiments indicated that the interaction between Gpr1 and Gpa2 depends on the presence of Plc1, while the interaction between Gpr1 and Plc1 does not require Gpa2, and the interaction between Plc1 and Gpa2 does not require Gpr1. Deletion of *GPR1*, *PLC1* or *GPA2* leads to defects of filamentation upon nitrogen starvation. These defects can be rescued by activating the cAMP pathway *via* overexpression of Tpk2 or by activating the MAPK pathway *via* an active *STE11-4* allele. Interestingly, Plc1 may crosstalk with the MAPK pathway. Deletion of Plc1 leads to a 5-fold reduction of the expression of the filamentation MAPK reporter *FG(TyA)::lacZ*, while deletion of Ras2 (an upstream regulator of the filamentaition MAPK pathway) only caused a 2-fold decrease. Perhaps Plc1 is downstream of Ras2 because dominant active $RAS2^{G19V}$ does not rescue the defect caused by Δ*plc1*. Gpr1 has no effect on *FG(TyA)::lacZ* expression, indicating that it

is not in the filamentation MAPK pathway (Ansari et al, 1999).

Gpr1 is required for pseudohyphal differentiation because a *gpr1Δ/gpr1Δ* mutant strain exhibited a significant defect in filamentous growth (Lorenz et al, 2000). Gpr1 is upstream of Gpa2 as demonstrated by the suppression of this growth defect with a constitutive $GPA2^{R237A}$ allele (supposed to induce decreased intrinsic GTPase activity) in a double Δ*gpr1Δras2* mutant (Xue et al, 1998). Expression of another dominant active $GPA2^{G132V}$ allele suppresses the filamentation defect of *gpr1Δ/gpr1Δ* mutant strains (Lorenz et al, 2000). Expression of Gpr1 is induced when cells are starved for nitrogen (Xue et al, 1998). However, neither nitrogen nor amino acids proved to be agonists for yeast Gpr1, though methionine might serve as ligand for the G protein coupled-receptor Gpr1 and Gpr4 from *C albicans* and *C. neoformans*, respectively (Lorenz et al, 2000; Maidan et al, 2005b; Xue et al, 2006). Like Gpa2, Gpr1 is essential for the transient rise in cAMP levels in response to glucose and over-expression of Gpa2 can not suppress the defect caused by Gpr1 (either deletion or none-function allele). These findings indicate that Gpr1 is a glucose sensor (Yun et al, 1998; Kraakman et al, 1999). Later on, other structurally related fermentable sugars including fructose, mannose and the disaccharide sucrose were found to be agonists of the Gpr1 receptor (Lorenz et al, 2000). Recently, by cysteine scanning mutagenesis and SCAM (substituted cysteine accessibility method), Gpr1 was demonstrated to directly interact with glucose and sucrose (Lemaire et al, 2004). It is a low-affinity glucose sensor and high-affinity sucrose sensor. In addition, mannose is an antagonist for both glucose and sucrose. However, fructose does not act on Gpr1 (Lemaire et al, 2004).

1.9.6 PKA: *BCY1* and *TPKs*; and Downstream of the cAMP Pathway

The regulatory subunit of the cAMP-dependent protein kinase (PKA) *BCY1* (bypass *CYR1*) gene was identified by a gene mutation that suppressed the *cyr1* mutation and bypassed the need for cAMP for growth (Matsumoto et al, 1982). The *bcy1* mutation caused a deficiency in the cAMP binding activity of a cAMP-dependent protein kinase and resulted in cAMP-independent activation (Matsumoto et al, 1982). Although yeast strains with *cyr1-* deletion and *ras1- ras2-* double deletion are not viable, strains *cyr1-bcy1* and *ras1-ras2-bcy1* are viable (Toda et al, 1985). The *BCY1* gene was cloned by complementation screening for strains sensitive to starvation and heat shock (Toda et al, 1987a). The Bcy1 protein is homologous to regulatory subunits of the mammalian cAMP-dependent protein kinase. Strains of *S. cerevisiae* with disrupted *BCY1* genes do not display a cAMP-dependent protein kinase activity *in vitro*, fail to grow on many carbon sources, and are exquisitely sensitive to heat shock and starvation (Toda et al, 1987a). All these phenotypes are the characteristics of the activated cAMP signalling pathway. *BCY1* also regulates pseudohyphal growth, because its disruption in diploid cells induces pseudohyphal differentiation and expression of Flo11, a cell surface flocculin which is a reporter for both the activated filamentation cAMP and MAPK pathways (Pan and Heitman et al, 1999; Rupp et al, 1999). The cAMP-dependent protein kinase is a tetrameric complex consisting of two regulatory subunits and two catalytic subunits. The regulatory subunits each contain two binding sites for cAMP. After binding to cAMP, the regulator subunits undergo conformational change that releases two active catalytic subunits with the regulatory subunits remaining as a dimer. Hydrolysis of cAMP by low affinity phosphodiesterases Pde1 and high affinity Pde2 restores PKA to the holoenzyme state (Borges-Walmsley and Walmsley, 2000).

Pde1 appears to down-regulate agonist-induced cAMP levels, while the high affinity Pde2 specifically controls the basal cAMP level in the cell. Activated PKA can phosphorylate Pde1 and render it much more efficient in cAMP hydrolysis, forming a feedback loop (Ma et al, 1999).

There are three different catalytic subunit genes (*TPK1*, *TPK2*, and *TPK3*) of cAMP-dependent protein kinase (PKA) from yeast (Toda et al, 1987b). The three Tpk proteins have conserved C-terminal residues and are homologous to mammalian catalytic subunits, but have heterogeneous N-terminal regions. Gene disruption experiments indicated that the three genes are redundant for viability but at least one *TPK* gene is required for normal cell growth (Toda et al, 1987b). Activated cAMP-dependent protein kinases have negative feedback on cAMP synthesis because a yeast strain with *TPK1/TPK2* deleted and *TPK3* partially inactivated showed high accumulation of cAMP on glucose-containing medium (Nikawa et al, 1987). The 3 Tpk proteins are not functionally redundant in cellular metabolism as demonstrated by genome-wide transcriptional profiling (Roberson et al, 2000). They also have different roles in regulation of pseudohyphal growth. Overexpression of Tpk2 stimulates pseudohyphal growth, while overexpression of Tpk1 and Tpk3 inhibit it (Pan and Heitman, 1999; Robertson and Fink, 1998). The Δ*tpk2*/Δ*tpk2* mutants are completely defective for pseudohyphal growth and exhibit a 10-fold reduction in the expression of Flo11. In contrast, deletion of *TPK1* and *TPK3* enhance pseudohyphal growth and Flo11 expression. The unique activating function of Tpk2 was mapped to the conserved catalytic region but not the unique N-terminal region (Pan and Heitman, 1999). By using yeast two-hybrid assays, two direct targets of Tpk2 are found to be the transcriptional activator Flo8 and transcriptional repressor Sfl1 (Pan

and Heitman, 1999; Robertson and Fink, 1998; Rupp et al, 1999; Pan and Heitman, 2002). Deletion of *FLO8* in the Δ*bcy1* strain abolishes pseudohyphal growth and Flo11 expression, while deletion of *SFL1* enhances pseudohyphal growth and Flo11 expression (Pan and Heitman, 1999; Robertson and Fink, 1998). Flo11 is the direct target of both Flo8 and Sfl1. The promoter of *FLO11* contains cis-element(s) in the -1400 to -1150 bp region for both Flo8 and Sfl1 binding. Phosphorylation promotes Flo8 binding and activation of Flo11, while phosphorylation inhibits Sfl1 binding and relieves the repression on Flo11 by unphosphorylated Sfl1 (Pan and Heitman, 2002).

An epistasis analysis indicated that PKA functions downstream of the Gpr1 receptor, Gpa2 and Ras2 proteins (Pan and Heitman, 1999; Borges-Walmsley and Walmsley, 2000). The PKA-activating *bcy1* mutation suppresses the filamentation defect caused by a Δ*gpa2*/Δ*gpa2* or Δ*gpr1*/Δ*gpr1* double deletion (Pan and Heitman, 1999). The Δ*tpk2*/Δ*tpk2* completely suppresses filamentation caused by the dominant active allele $GPA2^{G132V}$. On the other hand, the Δ*tpk2*/Δ*tpk2* can only partially suppress filamentation caused by dominant active $RAS2^{G19V}$, consistent with Ras2 signaling *via* both the cAMP and MAPK pathways (Pan and Heitman, 1999).

PKA signaling is independent of MAPK pathway, because activation of the filamentous growth by PKA does not require components of the MAPK pathway, such as Ste12 and Tec1, nor does it stimulate the expression of filamentation MAPK pathway reporter *FG(TyA)::lacZ*, although it stimulates the expression of Flo11 (Pan and Heitman, 1999). Flo11 is a cell surface flocculin responsible for cell adhesion (or flocculation) and agar invasion (Lo and Dranginis, 1996 and 1998). Flo11 is the

terminal target shared by both the cAMP pathway and filamentation MAPK pathway (Rupp et al, 1999). It is directly regulated by the transcription factors Flo8, Sfl1, Ste12 and Tec1. Ste12 and Tec1 are downstream elements of the filamentation MAPK pathway, while Flo8 and Sfl1 are elements of the cAMP pathway. Exogenous cAMP can bypass the requirement for the transcription factor Ste12, but not the requirement for Flo8, in the activation of Flo11 expression (Rupp et al, 1999). Adding cAMP to a *Δras1Δras2Δpde2Δste12* strain can trigger Flo11 expression, but can not stimulate the expression of the filamentation MAPK pathway reporter *FG(TyA)::lacZ*, indicating that Flo11 is the terminal target in the cAMP pathway (Rupp et al, 1999).

1.9.7 Summary of the cAMP-Signalling Pathway in *S. cerevisiae*

The cAMP signalling pathway that controls filamentous growth in *S. cerevisiae* is summarized in Figure 1.9 (adapted from Borges-Walmsley and Walmsley, 2000). The central component of the cAMP signalling pathway is adenylate cyclase which catalyzes the formation of cAMP. Adenylate cyclase is tightly bound to cyclase-associated protein CAP, which is essential for Ras activation and might play a role in subcellular localization of adenylate cyclase (Shima et al, 1997; Zelicof et al, 1996). Both guanine nucleotide-binding proteins Gpa2 and Ras activate adenylate cyclase, modulate cAMP levels, and regulate both vegetative and pseudohyphal growth (Borges-Walmsley and Walmsley, 2000). There are two RAS proteins in the yeast, Ras1 and Ras2. Ras2 is more potent than Ras1 in adenylate cyclase activation (Toda et al, 1985). The RAS proteins directly act on adenylate cyclase to maintain the basal cAMP level (Borges-Walmsley and Walmsley, 2000; Kido et al, 2002). The activation of adenylate cyclase by RAS proteins is dependent on GTP, farnesylation

and the N-terminus of CAP. RAS proteins are regulated by GEFs (guanine nucleotide exchange factors Cdc25 and Sdc25) and GAPs (GTPase-activating proteins Ira1 and Ira2; Thevelein and de Winde, 1999). The upstream components of Ras have not been identified but Ras mediate glucose signalling (Colombo et al, 2004). It is believed that Gα protein Gpa2 interacts directly with adenylate cyclase but there is no direct evidence for such an interaction. Gpa2 is coupled to the G protein-coupled receptor Gpr1 whose ligand is glucose or other fermentable sugars (Lamaire et al, 2004). The G protein complex in the cAMP pathway is not a canonical one. The kelch repeat protein Gpb2/Krh1 (or Gpb1/Krh2) and the Gγ mimic Gpg1 form a Gβγ mimic which interacts with Gpa2 and negatively regulates it. The Gβγ mimic also negatively regulates its own effector that is still unidentified (Harashima and Heitman, 2002 and 2005), but possibly adenylate cyclase (Hoffman, 2005a).

Binding of cAMP to the regulatory subunit Bcy1 of protein kinase A (PKA) results in the release of active catalytic kinase subunits Tpk1, Tpk2 and Tpk3, among which Tpk2 is the most important in stimulating pseudohyphal growth (Borges-Walmsley and Walmsley, 2000; Pan and Heitman, 1999). Tpk2 phosphorylates the transcription activator Flo8 and the transcription repressor Sfl1, both of which in turn trigger the expression of the cell wall flocculin Flo11 (Pan and Heitman, 2002). Activated PKA can also phosphorylate Pde1 and promote its efficiency in cAMP hydrolysis. Feedback regulation of the cAMP signalling pathway is controlled through PKA and Pde (Ma et al, 1999).

1.10 The G protein-MAP Kinase Signalling Pathway of *S. cerevisiae*: Mating and Pseudohyphal Growth

Haploid yeast cells exhibit two mating phenotypes, designated MATa and MATα. When MATa and MATα cells are co-cultured, they conjugate and form a third cell type, the diploid MATa/MATα cell (Versele et al, 2001). When the diploid cells are starved for both nitrogen and carbon nutrition, they undergo meiosis (Lengeler et al, 2000). The oligopeptide pheromone (a or α-factor) secreted by one mating type elicits three pheromone responses in the opposite mating type: division arrest at the G1 phase of the cell cycle; induction of pheromone-induced gene expression (e.g. *FUS1* transcription); and formation of an altered morphology termed shmooing or polarized morphogenesis (Versele et al, 2001; Dohlman and Thorner, 2001). These responses are mediated by the cell surface receptors encoded by *STE2* or *STE3* and the heterotrimeric G proteins encoded by *GPA1* (α subunit), *STE4* (β subunit) and *STE18* (γ subunit), respectively (Whiteway et al, 1989; Hoffman, 2005a).

The mating pheromone receptors Ste2 and Ste3 are similar to mammalian G-protein coupled receptors in that they have seven transmembrane domains. Although Ste2 and Ste3 lack significant sequence homology, they are all coupled to the Gα protein Gpa1 and signal division arrest and other mating responses by similar mechanism (Burkholder and Hartwell 1985; Hagen et al, 1986). *GPA1* was the first G protein cloned from yeast, using a cross-hybridization with a rat G protein (Nakafugu et al, 1987). The primary structure of Gpa1 is homologous to mammalian G protein α subunits but Gpa1 has an extra 110 amino acid residues near the N-terminal region. Gpa1 is localized to the membrane as it contains consensus signals for N-terminal myristoylation (Dohlman et al, 1993). Gpa1 is specifically expressed in haploid cells

and is essential for mating-factor-mediated signal transduction (Dietzel and Kurjan, 1987).

In the *S. cerevisiae* pheromone MAPK pathway, the G proteins are canonical αβγ heterotrimers. Genetic data indicated that oligopepeptide mating pheromones, a-factor and α-factor, bind to Ste3 and Ste2 respectively, and activate the heterotrimeric G proteins. Both Gpa1 and Ste4 (β) can interact with the C-terminal cytoplasmic domain of the pheromone receptor (Ongay-Larios et al, 2000). Functional coupling between receptors and G proteins is characterized by high-affinity binding of pheromone to its receptor in purified membrane preparations (Jenness et al, 1983; Blumer and Thorner, 1990). The α subunit Gpa1 is coupled with the receptor Ste2 or Ste3 in its GDP-binding state as indicated by high-affinity pheromone binding to its receptor. Gpa1 dissociates from its receptor upon binding GTP and this is indicated by decreased pheromone binding. The coupling between Gpa1 and its receptors requires the presence of both the β and γ subunits because pheromone binding to its receptor decreased when either the *STE4* or *STE18* genes were altered (loss-of-function for Ste18; loss-of-interaction with Gpa1 for Ste4). It is supposed that the yeast Gβγ dimer contributes to the interaction between the Gα subunit and the receptor by directly interacting with Gpa1 and causing a conformational change in this Gα subunit or by directly contacting the receptor itself (Blumer and Thorner, 1990). Indeed, the Gβ Ste4 can directly interact with both the pheromone receptor and the Gα Gpa1 in yeast two-hybrid analyses (Ongay-Larios et al, 2000). A Gβ mutation (L132F), which keeps the interaction with the pheromone receptor but abolishes the interaction with Gpa1, also disrupts the proper coupling of Gpa1 with the pheromone receptor (Ongay-Larios et al, 2000). Interestingly, this

$G\beta^{L132F}$ mutant (named *ste4-10*) is constitutive in activating the mating pathway but fails to respond to mating pheromone, indicating that the Gα protein is upstream of Gβγ in mediating the signal (Ongay-Larios et al, 2000). Although the receptor coupling of Gpa1 requires Gβγ, the association of Gpa1 with the membrane is independent of the β and γ subunits (Blumer and Thorner, 1990).

Upon pheromone stimulation, the Gα Gpa1 becomes GTP charged, uncoupling it from the receptor, and concertedly, releases the Gβγ dimer. In yeast, it is the Gβγ dimer that modulates downstream effectors, while Gpa1 negatively regulates the pheromone pathway. Loss of Gpa1 induces a pheromone response, while deletion of either Ste4 or Ste18 leads to an inability to activate the pheromone response, even when *GPA1* is absent (Whiteway et al, 1989). On the other hand, overexpression of *STE4* alone or together with *STE18*, generated a pheromone response in haploid cells, while cells with *GPA1* overexpression alone were more resistant to pheromone. Although overexpression of *STE18* alone had no pheromone response, *STE18* is essential for the pheromone response since overexpession of *STE4* (β) in the absence of *STE18* (γ) did not produce the pheromone response (Cole et al, 1990; Whiteway et al, 1990). These results suggest that the Gβγ dimer stimulates the pheromone signaling pathway, while the Gα Gpa1 plays a negative regulatory role. The function of Ste18 is to aid proper folding of Gβ Ste4 and localize it to the plasma membrane since it contains a conserved CAAX motif for palmitoylation or farnesylation of the cysteine residue (Garcia-Higuera et al, 1996).

The target of Gβγ is the conserved mitogen-activated protein kinase (MAPK) cascade, which includes Ste11, Ste7, and Fus3 (Lengeler et al, 2000). Free Gβγ

(Ste4 and Ste18) dimer directly interacts with Ste5, a scaffold protein holding the components of MAPK cascade together (demonstrated by yeast two-hybrid assay; Whiteway et al, 1995), and Ste20, a p21-activated protein kinase PAK-related protein kinase (demonstrated by immunoprecipitation; Leeuw et al, 1998). The membrane associated free Gβγ recruits Ste5 to the membrane, bringing Ste5 in proximity to the cell surface-associated Ste20 (Pryciak and Huntress, 1998). The interaction between Gβγ and Ste5 is constitutive and does not depend on pheromone activation, while the interaction between Gβγ and Ste20 depends on pheromone stimulation. Therefore, Gβγ forms a complex with the MAPK cascade through Ste5, and upon pheromone stimulation, binds and activates Ste20. Concurrently, activation of Ste20 may also require Cdc42, a Rho family GTPase, and Cdc24, a guanine nucleotide exchange factor for Cdc42 because Gβγ can directly interact with Cdc24 (Zhao, et al, 1995; Nern and Arkowitz, 1998; Nern and Arkowitz, 1999). Activated Ste20 phosphorylates the MAPKKK Ste11 (van Drogen et al, 2000), which then phosphorylates the MAPKK Ste7. Ste7 then phosphorylates and activates the MAPK Fus3, which activates the transcriptional factor Ste12 by phosphorylating the Ste12 repressors Dig1 and Dig2, leading to expression of genes required for mating (Madhani et al, 1997; Tedfork et al, 1997).

In yeast, the MAPK cascade is also required for pseudohyphal growth (Lengeler et al, 2000). In diploid cells, some of the same kinases and Ste12 in the haploid pheromone MAPK pathway are utilized for filamentous growth, but the pheromone receptors and the heterotrimeric G proteins are not expressed in diploid cells and are not required in pseudohyphal differentiation (Liu et al, 1993). The scaffold protein Ste5 is not required either. Strains with mutations in *STE7*, *STE11*, *STE12* and

STE20 have impaired pseudohyphal growth (Liu et al, 1993). There are two transcriptional reporters that have been used to discriminate between genes involved in the filamentous growth pathway and those in the pheromone mating pathway: they are *FG(TyA)::lacZ* and *FUS1::lacZ.* The *FG(TyA)::lacZ* was derived from transposon Ty1 whose transcription depends on *STE12* and *TEC1* (Laloux et al, 1994). Expression of *FG(TyA)::lacZ* correlates extremely well with the activation of the MAPK cascade in filamentous growth but does not correlate with the pheromone induced activation of the MAPK cascade. In contrast, the *FUS1::lacZ* reporter responds to pheromone stimulation but not the signals for activation of filamentous growth, such as nitrogen starvation (Trueheart et al, 1987; Hagen et al, 1991; Mosch et al, 1996).

Apart from stimulating the cAMP signaling pathway, Ras2 is also an upstream activator of the MAPK pathway for filamentous growth. The dominant active $RAS2^{G19V}$ gene simulates both filamentous growth and the expression of *FG(TyA)::lacZ* but not the mating pathway reporter *FUS1::lacZ* (Mosch et al, 1996). This activation is dependent on Cdc42, Ste20, the 14-3-3 proteins Bmh1 and Bmh2, a conserved MAPK pathway including Ste11 (MAPKKK), Ste7 (MAPKK), the MAPK Kss1 and the transcription factors Ste12/Tec1, but is independent of the components of cAMP signaling pathway ((Mosch et al, 1996; Cook et al, 1996; Madhani et al, 1997; Roberts et al, 1997). Neither deletion of *BCY1* nor overexpression of *TPK* genes enhances *FG(TyA)::lacZ* expression (Mosch et al, 1996).

The transcriptional factor Ste12 binds to pheromone response elements (PREs) that

have the consensus sequence TGAAACA (Dolan et al, 1989). This consensus sequence is also found in the reporter *FG(TyA)::lacZ.* Interestingly, Ste12 is the terminal component in both the pheromone and filamentous growth pathways (Madhani and Fink, 1997). The question is how the yeast cell discriminates between the two signals with just one transcription factor. In fact, another transcription factor Tec1 is also necessary for filamentous growth (Gavrias et al, 1996). Tec1 binds to a consensus sequence (TCS) CATTCT, which is also found in the filamentous growth pathway reporter *FG(TyA)::lacZ* and is only 14 base pairs away from the Ste12 PRE. In yeast cells, PRE and TCS compose a filamentation and invasion response element (FRE), which is called Ty1 promoter in the reporter *FG(TyA)::lacZ.* Tec1 binds the FRE in concert with Ste12 to promote the transcription of genes specifically required for filamentous growth (Madhani and Fink, 1997). One of the direct targets of Ste12/Tec1 is Flo11, a cell wall flocculin which is responsible for cell adhesion and agar invasion (Lo and Dranginis, 1998). The promoter region of *FLO11* for Ste12 and Tec1 binding is very similar to that in the filamentation MAPK pathway reporter *FG(TyA)::lacZ.* Flo11 is the terminal target of both the cAMP pathway and the filamentation MAPK pathway (Rupp et al, 1999). Deletion of *FLO11* abolishes both pseudohyphae formation in diploid cells and agar invasion in haploid cells (Lo and Dranginis, 1998).

The MAP kinase in the filamentaion MAPK pathway is Kss1, while Fus3 has an analogous role in the pheromone MAPK pathway (Madhani et al, 1997). In the filamentation MAPK pathway, Kss1, together with repressors Dig1 and Dig2, bind to and inhibit the Ste12/Tec1 dimer (Cook et al, 1996). Phosphorylation of Kss1 leads to the phosphorylation of Dig1 and Dig2. The phosphorylated Kss1, Dig1 and Dig2

dissociate from the Ste12/Tec1 and relieve repression, leading to activation of Flo11 transcription (Bardwell et al, 1998).

1.11 Cross Talk between Distinct Signalling Pathways Regulates Filamentous Growth

The cAMP pathway and MAPK pathway independently regulate filamentous growth. Exogenous cAMP restores filamentaton in *ste20* and *ste12* mutant strains but does not stimulate the expression of the MAPK reporter *FG(TyA)::lacZ* (Lorenz and Heitman, 1997). The cAMP pathway is responsible for unipolar budding and invasion, while the MAPK pathway is required for cell elongation and invasion (Pan and Heitman, 1999). The *Δtpk2/Δtpk2* mutant strains looses the unipolar budding pattern and agar invasion but retains the ability for cell elongation, while *Δste12Δste12* mutants do not display cell elongation and agar invasion but still undergo unipolar budding (Pan and Heitman, 1999).

There is cross talk between the cAMP pathway and the filamentation MAPK pathway. First, Ras2 has been implicated in both pathways. In addition to its well-established role in regulating adenylate cyclase activity, $Ras2^{G19V}$ stimulates the MAPK cascade and the reporter *FG(TyA)::lacZ* (Mosch et al, 1996). Secondly, the two pathways converge on the cell wall flocculin Flo11, which is responsible for cell adhesion and agar invasion under nitrogen starvation (Lo and Dranginis, 1996; Lo and Dranginis, 1998; Rupp et al, 1999). The direct regulators of Flo11 are Flo8, Sfl1 (both are in the cAMP pathway), Ste12 and Tec1 (transcriptional activators in the filamentation MAPK pathway). Flo11 has a higher expression level in haploid cells than in diploid cells. This explains why haploid cells invade agar in rich media while

the diploid cells can not. The primary structure of Flo11 is similar to a class of yeast serine/threonine-rich GPI (glycosyl-phosphatidyl-inositol)-anchored cell wall proteins. Overexpression of Flo11 enables agar invasion in rich media, while deletion of Flo11 abolishes pseudohyphal formation and agar invasion. Flo11 is a good reporter for both the cAMP pathway and the filamentation MAPK pathway. Activation of either pathway increases Flo11 expression, while mutations in either pathway blocks Flo11 expression. The promoter of the *FLO11* gene contains cis-elements for the binding of transcriptional factors Flo8, Sfl1, Ste12 and Tec1. Overexpression of Flo8 in a Δ*ste12* strain and overexpression of Ste12 in a Δ*flo8* strain suppressed the pseudohyphal and invasion defects of the mutants. Interestingly, the morphologies of the filaments of these suppressed strains were not identical to that of the wild type. In the Δ*ste12* strain with Flo8 overexpression, the cells were shorter but had a denser network. In the Δ*flo8* strain with Ste12 overexpression, the cells were longer than the filaments of the wild-type (Lo and Dranginis, 1996; Lo and Dranginis, 1998; Rupp et al, 1999). These observations are consistent with the conclusion that the cAMP pathway regulates unipolar budding and invasion, while the MAPK pathway is essential for cell elongation and invasion (Pan and Heitman, 1999). Meanwhile, these observations indicate that terminal structural components other than Flo11 exist and are under the control of the cAMP pathway and the filamentation MAPK pathway, respectively.

1.12 Other Genes Regulating Pseudohyphal Growth

A number of other genes have been reported to regulate pseudohyphal growth. These include the protein kinases *ELM1*, *ELM2*, *ELM3* (Blacketer et al, 1993), *SCH9* (Xue et al, 1998), and the transcription factors *PHD1* (Gimeno and Fink, 1994),

ASH1 (Chandarlapaty and Errede, 1998), *SWI5*, *SOK2* (Pan and Heitman, 2000), *MSN2*, *MSN4* (Pan and Heiman, 1999) and *MSS11* (Gagiano et al, 2003). Elm1p is a serine-threonine protein kinase and its function is to repress filamentous growth (Koehler and Myers, 1997). However, Elm1 is not an element in either the cAMP/PKA pathway or the MAPK pathway. Overexpression or deletion of the *ELM1* gene can complement the abnormal phenotypes in dimorphism caused by mutations in the cAMP signaling pathway (Garret, 1997). Sch9 is a protein kinase under the control of Gpr1 and Gpa2 but is parallel with Ras and the cAMP pathway (Xue et al, 1998; Lorenz et al, 2000). Phd1 is a transcription factor, overexpression of which induces invasive pseudohyphal growth on nutritionally rich medium. Phd1 acts distinctly from the cAMP pathway, MAPK pathway and Ash1, which positively regulate filamentous growth (Chandarlapaty and Errede, 1998). Ash1 is proposed to be a transcription factor because it contains a zinc finger-like domain related to the GATA family of transcription factors. Ash1 appears to be under the control of Ras2 and Cdc42 but is not a component of the MAPK pathway. Mutants with deletion of *STE11*, *STE7* and *STE12* can still form filaments if they express the activated alleles $Ras2^{G19V}$ or $Cdc42^{12V}$. Further deletion of *ASH1* blocks filamentous growth. In addition, overexpression of Ash1 can restore the defect of filamentous growth caused by *ste7*, *ste11*, *ste12* and *ste20* deletion, but has no effect on the filamentation MAPK pathway reporter *FG(TyA)::lacZ* (Chandarlapaty and Errede, 1998). Swi5 is a zinc finger class transcriptional factor that can regulate Ash1 expression (Pan and Heitman, 2000). The transcription factor Sok2 negatively regulates pseudohyphal growth through a complex cascade of Phd1, Swi5 and Ash1. The *sok2* mutation enhances expression of Phd1, Ash1, and Swi5 genes. Phd1, Ash1 and Swi5 all activate the *FLO11* gene independently of the cAMP and MAPK pathways (Pan and

Heitman, 2000).

1.13 The cAMP Signalling Pathway of *Schizosaccharomyces pombe*

In contrast to *S. cerevisiae*, mating in the fission yeast *S. pombe* is regulated by both the pheromone MAPK pathway and nutrient-sensing cAMP pathway (Lengeler, et al, 2000). The pheromone MAPK pathway is similar to that of *S. cerevisiae* but there are still a number of differences (Hoffman, 2005a). Perhaps the most obvious is that the G protein in the MAPK pathway of *S. pombe* is not a typical heterotrimeric complex because Gpa1 appears to be monomeric. The Gβγ dimer (Git5-Git11) is not the cognate partner for Gpa1 but they are coupled to Gpa2 to form a typical heterotrimeric G protein in the cAMP signaling pathway. In contrast to *S. cerevisiae*, it is Gpa1 in *S. pombe* that activates the pheromone MAPK pathway. In addition, Ras1 activates the pheromone MAPK pathway but does not regulate the cAMP pathway. For details of the pheromone MAPK pathway of *S. pombe*, please refer to review by Hoffman (2005a).

The cAMP signaling pathway in *S. pombe* is responsible for mating, glucose sensing and glucose repression on gluconeogenesis (Hoffman, 2005b). Glucose detection results in elevated cAMP levels and activation of the cAMP signaling pathway. The transcription of the *fbp1* gene, which encodes fructose-1,6-bisphosphatase, is repressed upon glucose stimulation, serving as a good reporter for activation of the cAMP pathway (Hoffman and Winston, 1990; Hoffman and Winston, 1991). Increased activity of the cAMP signaling pathway inhibits sexual development, while decreased activity leads to conjugation and sporulation (Lengeler et al, 2000). The major components of the cAMP signaling pathway include the seven-transmembrane

GPCR Git3, the heterotrimeric G-protein composed of the Gpa2 Gα (Git8) and Gβγ (Git5-Git11) dimer, the adenylate cyclase Cyr1 (Git2) and Pka1 (Git6), the catalytic subunit of cAMP-dependent protein kinase A (PKA; Hoffman, 2005b). The GPCR Git3 interacts with Gpa2 in two-hybrid assays. The interaction is facilitated by the Gβ Git5 and abolished by a dominant active Gpa2 (Hoffman, 2005b). The Gγ Git11 was obtained from a two-hybrid screening with Gβ Git5 (Landry and Hoffman, 2001). Gpa2 functions downstream of GPCR and Gβγ because the defect of *fbp1* transcriptional repression caused by Δ*git3*, Δ*git5*, or Δ*git11* is suppressed by overexpression of the wild type *gpa2* or an activated $gpa2^{R176H}$ allele. Deletion of Gβγ leads to the same phenotype as that of Gpa2 deletion (Welton and Hoffman, 2000; Landry and Hoffman, 2001). This is in contrast to the *S. cerevisiae* pheromone signaling pathway in which deletion of Gpa1 leads to the opposite phenotype of Ste4-Ste18 Gβγ deletion.

Gpa2 is the direct activator of adenylate cyclase (Ivy and Hoffman, 2005). The adenylate cyclase gene of *S. pombe*, *spCYR1*, was cloned by cross-hybridization with *S. cerevisiae CYR1* (Yamawaki-Kataoka et al, 1989; Young et al, 1989). The protein consists of 1,692 amino acids and a segment corresponding to the N-terminal 620 residues of *S. cerevisiae* Cyr1 appears to have been lost. When analyzed by SMART EMBL, *spCYR1* contains a RA domain (292-372), LRR domains (475-876), a PP2Cc domain (985-1273) and a catalytic CYCc domain (1261-1506). Unlike *S. cerevisiae*, there is no indication that the *S. pombe* adenylate cyclase is regulated by Ras1 although there is an obvious RA domain (Hoffman, 2005a). Interestingly, a recent discovery indicates that the RA domain of spCyr1 binds directly to GTP-charged Gα Gpa2 and plays an important role in adenylate cyclase activation (Ogihara et al,

2004). In another report, however, the interaction domain of adenylate cyclase with Gpa2 was mapped with a two-hybrid screen to residues 33-196, in which the L177, T178, P180 and P182 are crucial for the interaction (Ivey and Hoffamn, 2005). Overexpression of this domain leads to a defect in cAMP signaling, which can be restored by Gpa2 coexpression. These are the first evidence for direct activation of fungal adenylate cyclase by a G protein.

1.14 The cAMP Signalling Pathway of *Candida albicans*

C. albicans is the most important opportunistic fungal pathogen in human beings (Lo et al, 1997; Brown and Gow, 1999). It causes a wide range of diseases from superficial infections to life-threatening systemic infections in immuno-compromised patients. This fungus is dimorphic. *Candida* yeast cells can undergo two different types of switching, i.e. pseudohyphal and hyphal switching. A pseudohypha is a chain of distinct cells formed by budding but without the separation of daughter cell and mother cell in each cell division. A hypha is formed from a germ-tube which is a cylindrical outgrowth from a yeast cell. The germ-tube extends to form a hypha, a long continuous tube with septa throughout the length, but no distinct indentation at the site of septum (Lo et al, 1997; Brown and Gow, 1999). Hyphal or pseudohyphal formation underlies pathogenesis because *Candida* variants defective in hyphal formation are less virulent (Sobel et al., 1984). Pseudohyphal and hyphal formation in *Candida* can be induced by many environmental factors. The most critical condition for pathogenicity may be the induction of hypha formation by biological conditions such as temperature change, serum and macrophages (Lo et al, 1997).

At least two signal transduction pathways, the MAPK pathway and cAMP pathway,

are involved in the morphological switching (Borges-Walmsely and Walmsley, 2000). The major components of the MAPK pathway include the kinases Cst20 (Ste20 homologue), Ste11, Hst7 (Ste7 homologue), Cek1(Kss1 homologue) and the transcription factor Cph1 (Brown and Gow, 1999); whereas the major components of the cAMP pathway include CaGpr1 (Miwa et al, 2004; Maidan et al, 2005a), CaRas1 (Feng et al, 1999; Leberer et al, 2001), CaGpa2 (Sanchez-Martinez and Perez-Martin, 2002), CaCdc35 (adenylate cyclase; Rocha et al, 2001), CaCap1 (Bahn and Sundstrom, 2001), CaTpk1, CaTpk2 (Bockmuhl et al, 2001; Cloutier et al, 2003), CaBcy1 (Cassola et al, 2004) and the transcription factor Efg1 (Lo et al, 1997). Interestingly, the transcription factor Cph1 is a homologue of Ste12, and Efg1 is a homologue of Phd1 in *S. cerevisiae* (Lo et al, 1997). Similar to *S. cerevisiae*, both *cph1/cph1* and *efg1/efg1* single-mutant strains are partially defective in filamentous growth. The double mutant *cph1/cph1*, *efg1/efg1* strain is almost completely defective in pseudohyphal and hyphal formation under any conditions tested, including serum and macrophage stimulations (Lo *et al.*, 1997). Most importantly, the non-filamentous cells show a drastic reduction in virulence when injected in mice (Lo *et al.*, 1997).

An increased cAMP level accompanies the yeast to filamentous transition (Sabie and Gadd, 1992). Adenylate cyclase CaCdc35 is essential for filamentous growth (Rocha et al, 2001) and cAMP levels are undetectable in a *CaCDC35* deletion strain. In contrast to *S. cerevisiae*, cells with *CaCDC35* deleted are viable but grow more slowly than wild type cells and are unable to switch to the hyphal form under all environmental conditions tested. Importantly, the homozygous *Cacdc35Δ* cells are avirulent for systemic infection in a mouse model (Rocha et al, 2001). The adenylate

cyclase CaCdc35 is regulated by Ras1 and Gpa2 (Leberer et al, 2001; Miwa et al, 2004; Maidan et al, 2005a), the later of which is coupled to the cell surface receptor CaGpr1 as indicated by two-hybrid assays (Maidan et al, 2005a). Both CaGpr1 and CaGpa2 are involved in hyphal formation in solid media in a cAMP-dependent manner, but they are not required for hyphal formation in liquid serum media or systemic candidiasis (Miwa et al, 2004). There is still a dispute as whether CaGpr1-CaGpa2 mediates glucose-induced signaling (Miwa et al, 2004; Maidan et al, 2005a). In contrast, CaCdc25-CaRas1 mediates glucose, serum and macrophage-induced cAMP signaling (Feng et al, 1999; Leberer et al, 2001; Maidan et al, 2005). A *Caras1Δ* strain is viable but can not form hyphae in response to serum, whereas a dominant active $CaRAS1^{G19V}$ allele enhances hyphal growth (Feng et al, 1999; Maidan et al, 2005a). The defect caused by the *Caras1*Δ can be restored by addition of cAMP in growth media or overexpressing components of the MAPK pathway, indicating that CaRas1 regulates both the cAMP and MAPK pathways. In addition, the filamentation defect caused by *Caras1*Δ is associated with reduced virulence in the mouse model (Leberer et al, 2001). There are two cAMP-dependent protein kinase catalytic subunits in *C. albicans*, Tpk1 (named Tpk3 later) and Tpk2 (Bockmuhl et al, 2001). Tpk1 controls the hyphal switch on solid inducing media, whereas Tpk2 controls agar invasion and the hyphal switch in liquid media. The conserved C-terminal catalytic portions mediate the Tpk specificities with regard to filamentation, while the N-terminal domain of Tpk2 mediates agar invasion.

1.15 The cAMP Signalling Pathway of *Ustilago maydis*

Ustilago maydis is the causative agent of corn smut disease. The disease is characterized by large plant tumors that are filled with masses of black-pigmented

telispores (Durrenberger et al, 1998; Lengeler et al, 2000). The life cycle of this fungus can be mainly divided into two parts: the *in vitro* yeast-like haploid cells and *in planta* dikaryotic hyphae. The infectious dikaryotic hyphae are formed after mating of two haploid cells with different alleles at the unlinked mating type loci *a* and *b*. The *a* locus encodes pheromones and pheromone receptors and mediates cell recognition and fusion. The *b* locus is multiallelic and encodes two homeodomain proteins bE and bW, which form heterodimers only if they are derived from different *b* alleles. The bE-bW heterodimer controls invasion and filamentous growth after fusion of two haploid cells. The mating, filamentous growth and virulence of this fungus are inter-correlated. In addition, haploid cells can also adopt filamentous growth under certain conditions such as nutrient starvation, exposure to air, or growth at low pH. However, haploid cells are not pathogenic (Durrenberger et al, 1998; Lengeler et al, 2000).

The cAMP pathway of *U. maydis* is involved in mating, filamentous growth and pathogenicity (Gold et al, 1994). The major components of the cAMP pathway include the Gα protein Gpa3 (Regenfelder et al, 1997; Kruger et al, 1998), the Gβ protein Bpp1 (Muller et al, 2004), adenylate cyclase Uac1 (Gold et al, 1994; Kruger et al, 1998), the PKA regulatory subunit Ubc1 (Gold et al, 1994; Gold et al, 1997), the PKA catalytic subunits Adr1 and Uka1 (Durrenberger et al, 1998). In contrast to *S. cerevisiae*, it is a decreased level of cAMP or decreased activity of the cAMP pathway that leads to filamentous growth (Lengeler et al, 2000). Defects of *uac1* in haploid cells can lead to filamentous growth without the requirement for mating (Gold et al, 1994). However, co-infection of maize seedlings with two *uac1*-deficient strains harboring compatible *a* and *b* mating-type alleles does not results in

disease, indicating that filamentation and virulence are divergently controlled (Mayorga and Gold, 1998). There are 4 Gα proteins in *U. maydis* (Gpa1-4). However, only deletion of *gpa3* has a discernable phenotype (Regenfelder et al, 1997). Gpa3 is essential for mating and pathogenicity. Deletion of *gpa3* causes mating deficiency in haploid cells and loss of pathogenicity in diploid cells, and leads to a morphological change similar to that caused by deletion of *uac1* (Regenfelder et al, 1997). The sterility and morphology change caused by *gpa3*Δ can be rescued by exogenous cAMP, indicating that *gpa3* is upstream of *uac1* (Kruger et al, 1998). Recently, the Gβ subunit *bpp1* was identified (Muller et al, 2004); and its deletion shown to cause filamentous growth and attenuated pheromone gene expression, the same phenotypes as the *gpa3*Δ strain. A dominant active allele of *gpa3* suppresses the phenotypes of the *bpp1* deletion strain, indicating that *gpa3* and *bpp1* are subunits of the same heterotrimeric G protein. In contrast to the *gpa3*Δ strain, the *bpp1*Δ strain has no deficiency in inducing plant tumors, indicating that *gpa3* acts independent of *bpp1* in pathogenic development (Muller et al, 2004). Of the two PKA catalytic subunits, Adr1 accounts for the majority of PKA activity, regulates filamentation and is essential for pathogenicity. In contrast, *uka1* plays little role in filamentation and pathogenicity (Durrenberger et al, 1998). Although Ras2 was identified in a screen for suppressor mutations of an *adr1* mutation, there is no indication that this Ras protein is involved in the cAMP signaling pathway. However, Ras2 promotes filamentous growth and pathogenicity through the MAPK pathway (Lee and Kronstad, 2002).

1.16 The cAMP Signalling Pathway of *Cryptococcus neoformans*

Cryptococcus neoformans is a significant human fungal pathogen that primarily

infects immunocompromised patients such as those with AIDS, malignancies, receiving chemotherapy or transplantation (Pukkila-Worley and Alspaugh, 2004). *C. neoformans* exists in the environment predominantly as haploid yeast with two mating types, MATa and MATα. They duplicate by budding and mate under the regulation of nutrient limitation and pheromones (Lengeler et al, 2000). Diploid cells are unstable and only occur transiently (Borges-Walmsley and Walmsley, 2000; Pukkila-Worley and Alspaugh, 2004). Diploid cells are filamentous and undergo meiosis and sporulation. MATα cells can also filament and sporulate in response to nitrogen starvation in the absence of MATa cells or in response to pheromone secreted by MATa cells (Lengeler et al, 2000). This perhaps makes MATα more prevalent in the environment and clinical isolates. In addition, MATα cells are more virulent than MATa cells (Lengeler et al, 2000). In *C. neoformans*, morphological switching is not critical to its pathogenicity; which depends mainly on three factors, production of antiphagocytic polysaccharide capsule and anti-oxidant melanin, and the ability to grow at 37°C (Pukkila-Worley and Alspaugh, 2004). The production of capsule and melanin is induced by signals encountered in the host during infection. For example, capsule formation can be induced by iron deprivation or physiological concentrations of CO_2/HCO_3^- (Vartivarian et al, 1993). Melanin production can be induced by glucose deprivation and presence of diphenolic substrates such as catecholamines (Nurudeen and Ahearn, 1979).

The mating, virulence and pathogenicity of *C. neoformans* are controlled by the cAMP signaling pathway (Pukkila-Worley and Alspaugh, 2004). The major components of the cAMP signaling pathway include the G protein-coupled receptor Gpr4 (Xue et al, 2006), Gα protein Gpa1 (Alspaugh et al, 1997), adenylate cyclase

Cac1 (Alspaugh et al, 2002), the PKA regulatory subunit Pkr1 and catalytic subunit Pka1 (D'Souza et al, 2001). Gpr4 is the *S. cerevisiae* Gpr1 homologue. It physically interacts with Gpa1 as indicated by the split-ubiquitin system. Deletion of *GPR4* leads to reduced capsule production and mating defects, similar to deletion of *GPA1* (Xue et al, 2006). Δ*gpa1* mutant strains are sterile and deficient in capsule and melanin production (Alspaugh et al, 1997). The adenylate cyclase gene *CAC1* is not essential for growth. Deletion of *CAC1* has no effect on viability and vegetative growth. However, deletion of *CAC1* leads to the same phenotypes as *GPA1* deletion (Alspaugh et al, 2002). Both *gpa1* and *cac1* mutants are non-pathogenic in a mouse model (Alspaugh et al, 1997 and 2002). Deletion of the PKA catalytic subunit *PKA1* leads to similar phenotypes to deletion of *GPA1* and *CAC1*, while the PKA regulatory subunit *pkr1* mutation suppresses *gpa1* capsule and melanin defects. The *pkr1* mutant strains are hypervirulent with more capsule produced than wild-type strains (D'Souza et al, 2001). The Gβ subunit gene *GPB1* has also been identified (Wang et al, 2000). However, Gpb1 regulates mating and haploid fruiting via the MAPK pathway. Gpb1 does not mediate virulence production and does not interact with Gpa1. The partner of Gpa1 has still not been determined.

Chapter II

Materials and Methods

2.1 Chemicals, Antibiotics and Solutions

General chemicals, antibiotics and biological chemicals were mainly purchased from Sigma, Fluka, BDH, and Merck. Microbial growth media were acquired from Oxoid, Difco and Clontech. Enzymes used in molecular biology were supplied by Promega, Roche Biochemicals and Invitrogen. A list of frequently used antibiotics and solutions is presented in Table 2.1. Other solutions were prepared as previously described (Sambrook et al, 1989).

Table 2.1 Antibiotics and solutions.

	Stock Concentration	Storage Condition	Working Concentration
Ampicillin	100 mg/ml	-20°C	100 μg/ml
Carbenicillin	100 mg/ml	-20°C	100 μg/ml
Chloramphenicol	30 mg/ml in ethanol	-20°C	30 μg/ml
Kanamycin	10 mg/ml	-20°C	20 μg/ml
Tetracyclin	5 mg/ml in ethanol	-20°C	10 μg/ml
IPTG	200 mg/ml	-20°C	4-12 μl for surface spread on 90 mm plate
X-Gal	50 mg/ml in dimethylformamide	-20°C	35 μl for surface spread on 90 mm plate
RNase A	10 mg/ml	-20°C	
DNase I	500 units/ml	-20°C	
Lyticase	10 units/ml in TE pH 7.5	-20°C	
Proteinase K	50 μg/ml	-20°C	

2.2 Strains, Media and Vectors

Pb01 strain (ATCC 90659) was purchased from ATCC USA. It is a typical wild type *Pb* strain which undergoes a morphological switch in response to a change in temperature. Other bacterial and yeast strains used in this thesis are summarized in Table 2.2-1. Culture media used for growth of strains are listed in Table 2.2-2. Vectors used in this thesis are listed in Table 2.2-3.

Table 2.2-1 Strains used in this thesis.

Strains	Source	Application
Pb01 (*P. brasiliensis*)	90659 from ATCC	DNA and RNA extraction
E. coli NovaBlue	Novagen	Competent cell for blue/white selection
E. coli XL1-Blue MRF'	Stratagene	Host of λ ZAP phage
E. coli XL10-Gold	Stratagene	Ultra-competent cell
E. coli SOLR	Stratagene	Phagemid excision
E. coli DH5α	Invitrogen	Competent cell for cloning
E. coli BL21 (DE3)	Novagen	Protein expression
E. coli BL21 (DE3) pLYS	Novagen	Protein expression
E. coli Rosetta 2 (DE3)	Novagen	Protein expression
E. coli Rosetta 2 (DE3) pLYS	Novagen	Protein expression
S. cerevisiae AD1-8$^-$	From Prof A Goffean	DNA extraction
S. cerevisiae AH109	Clontech	Yeast two hybrid

Table 2.2-2 Culture media used in this thesis.

Media	Composition of media	Application
LB	10 g/L NaCl, 5 g/L yeast extract, 10 g/L bactotryptone	*E. coli* culture
YPD	20 g/L Difco peptone, 10 g/L yeast extract, 2% Glucose	Yeast culture
Modified-YPD	20 g/L neopeptone, 10 g/L yeast extract, 2% Glucose	*Pb01* culture

Table 2.2-3 Vectors used in this thesis.

Vectors	Source	Application
pGEM-T Easy	Promega	TA cloning
pGEM-TE(*Kpn*I)	pGEM-T Easy introduced with a *Kpn*I site	Cloning of full length *PbCYR1*
pBSK	Invitrogen	Cloning
pYES6/CT	Invitrogen	Cloning and expression
pDNR-LIB	Clontech	*Pb01* cDNA library construction
pGADT7	Clontech	Yeast two hybrid
pGBKT7	Clontech	Yeast two hybrid
pET-21a	Novagen	Protein expression

2.3 Storage of Strains

All the operations with *Pb01* were carried out in two especially established Category 3 labs (UK category for infectious agents) in the IBLS (Institute of Biological and Life Science), University of Glasgow, and in the CID (Centre for Infectious Diseases) at Durham University. Long term storage of *Pb01* was achieved by maintaining the cells in 15% glycerol at -70°C. *Pb01* yeast form cells were grown into log phase (3-4 days) in 50 ml modified YPD medium with shaking at 120 rpm.

Replicate stocks were prepared by mixing 0.5 ml of cells and 0.5 ml of 30% sterile glycerol. The mixture was then placed in a -80°C freezer. Other bacterial and yeast cultures were stored in a similar manner. Usually, a 5 ml overnight liquid culture was propagated. Stocks were prepared by mixing 0.75 ml of the culture with 0.25 ml 60% glycerol. The appropriate storage vials containing this mixture were then placed at -80°C.

2.4 Centrifugation

Routine centrifugation of 1.5-ml and 0.5-ml tubes was performed in a benchtop Eppendorf Centrifuge 5415D. Routine centrifugation of 20-ml universal tubes, 15-ml and 50-ml Falcon tubes were carried out in Jouan CR3i benchtop centrifuge. For centrifugation of larger volume or with higher speed, Beckman's Avanti J-E centrifuge was employed, with either JA-20 or JA-10 rotors being used.

2.5 DNA Agarose Gel Eletrophoresis

As previously described (Sambrook et al, 1989), DNA agarose gel electrophoresis was routinely performed with 1 X TAE buffer (NBS Biologicals, UK).

2.6 PCR Techniques

PCR was a frequently used technique in this thesis for cloning genes and making plasmid constructions. Four major PCR systems were used in this thesis. They are Qiagen's HotStarTaq and ProofStart Taq, Promega's Taq in storage buffer B, ABgene's Extensor High-Fidelity PCR system, and Stratagene's Gene Morph II Random Mutagenesis PCR System.

2.6.1 Oligonucleotide Primers for PCR

Oligonucleotide primers were synthesized by Invitrogen. Primers were reconstituted in distilled water to a concentration of 50 pmol/μl. The primers used in this thesis are listed in Table 2.6.1.

Table 2.6.1 Primers used in this study.

Primers	**Sequences** (5'→3'; restriction sites are underlined; A+C+G=V, T+C+G=B, A+T+G=D, A+T+C=H, A+T=W, C+G=S, T+G=K, A+C=M, C+T=Y, A+G=R, A+T+C+G=N)
PbCYR1	
PbAC-F1	GTBTTCACYGAYATYAAGAACWSBAC
PbAC-F2	AGAACTGCACGTCGCTTTGG
PbAC-F3	GGAAGCTGAAGGGGCTTGAG
PbAC-F4	AGTTGCGGATGGTGGGCAAATC
PbAC-F5	CCGTCCTCGAGGTGCAATGGAAACCCCATCCTCG
PbAC-F6	TATGGCAGATAGCCTTGGGCGAAAC
PbAC-F7	CAATCTGAAGAGTCTCGTTGATCTTGATATC
PbAC-F8	GTGCCCTGAAAATTTCGGAGAGATTGGTCTTC
PbAC-F9	GTCTGGCGCCGTTGGACTGTTGCTTTACGTTTGG
PbAC-F10	GCCTCAACGCTCCACGGGCTGAGATTGAAGAGG
PbAC-F11	CCGTTGCCTATGACTTGATGTC
PbAC-F12	CCAGACACTCGTCGTCCTCAAAGAGTA
PbAC-F14	GAGAATTCCGCAATCCCTTTGAACCCTCCAG
PbAC-F15	CGTTTACGTTTGCGGGATCTCTGAAGCAAC
PbAC-F16	GAATATCGGCCGGATGAATCACATCCACTC
PbAC-F17	AGCGGCCAGAAACTTAGATTTGCGGCAGCA
PbAC-F18	CAGCATGGCAAGGAGACAGCGGGAGAAAGA
PbAC-F19	GAGATGCACCTGTGTATCCAGTTCCAGGAGA
PbAC-F20	CCCCAACCCGGGATTTGAGCTACTCACAGA or TCCACGTCGCTTTGGGAGACGTATCC
PbAC-F21	TTGGGATACCGTCTGCTCTTCCTCTCGCCA
PbAC-F22	AGAGAGTCCCGTGCGCTGACACAGCCTTGA
PbAC-F23	TTGTCACCGCGAGTTAAAGTCCGGAGTTGA
PbAC-F24	CCACGTCGACTTGCACGGGCGCAGTCTCA
PbAC-F25	GTCATATCTCGAGCTGCAACTGAAACCCCA
PbAC-F26(KpnI)	GGTACCAAAATGTCTAGGAGACAGCGGGAGAAAGATAGG

PbAC-F27	AGATTTCGGATCAGGATGACAGAAGGCTGA
PbAC-F28	GATTTCGGATCAGGATGACAGACACTCGTC
PbAC-F40	CCGCATTTCCGCAGTTGCGGATGGTGGGCAAATC
PbAC-GSP1	TGATGCTGTTGAATCGTGCGTCTATCTC
PbAC-R1	TTVACCATRGGRCCSWRGATRTCCAT
PbAC-R2	CAAGTAATCCATTCGACCAG
PbAC-R3	CCGTCAACTCGTTATAGGACAAG
PbAC-R4	NNNNCTCGAGTGGCGGCCGCTCTAGAACTA
PbAC-R5	NNNNCTCGAGGTCTATAATATCTAATAGTGATG
PbAC-R6	CAGGATTCTCAAGCCCCTTCAGCTTC
PbAC-R7	AGTCACCCATAACCGTTCCAGTGTCGTAAGGCGTC
PbAC-R8	TTCCAGTGTCGTAAGGCGTCCAATATCCGACAACTC
PbAC-R9	ACTCACCTCCACCCGCGTAACCTGATGTTC
PbAC-R9(2)	GTTGAAGCTGATATCAAGATCAACGAGACTCTTC
PbAC-R10	GTCCAGAGTTCTTGAAAGGTC
PbAC-R11	CGCAGACTCCTTTTTGCGGTCAGACTCGACAC
PbAC-R12	AGCTCGAATTCCGACCATATCTAGCAGGAGAC
PbAC-R13	TCAAAAACCTAGATCCCTTCCCACCACCGTCG
PbAC-R14	GATTGACTGCCAGATGACATAGG
PbAC-R15	GTGGTGTCCATGATGGCGACCGCTTCC
PbAC-R16	GTCATCCGATGCGCCAAGAATCGCAAG
PbAC-R17	CCAAAGCTGGTTGTCGGTACGTCTGTTG
PbAC-R18	CAGAGGAGAGAACTTGGCATTTCCGAACCA
PbAC-R19	TATCTGAGCCAACTGTCGACGGAAGAGATC
PbAC-R20	TCCAAAGCTGGTTGTCGGTACGTCTGTTGG
PbAC-R21	TGGGGATACATCGGGGGAATGTGGCCTAC
PbAC-R22	GAGTCACGCTCGCCATCTGTCATGGAACCA
PbAC-R23	CTCTTTGAGGACGACGAGTGTATTTTGAAC
PbAC-R24	AAGAAACCCCCAAGCAGCATGAAGCAGGCA
PbAC-R25	ACCCATCCAAGCCCGAAAGAGCAACGTCCA
PbAC-R26	GCCAAACAGCGAAACAACGCCGTTCCGGCA
PbAC-R27	GGCGTTACGATTACTCTTTGAGGACGACGA
PbAC-R28	GAATGTGGCCTACGGGGGGCGTTACGATTA
PbAC-R29	CTGGTTGTCGGTACGTCTGTTGGGGATACA
PbAC-R30	CCCTGCGGAATCTCCGTCAACTCGTTATAGGACAAG
PbAC-R31	GACGGGGGGTTGAAGCTTTCGCCTCTCACA
PbAC-R32	AACAATATCGATCGTACCAAGCAAACAGACGCA
PbAC-R33	CAAATCCACCCATATGAGCCAACTGTCGAC
PbAC-R330	CATACTGTGGCCTGCAAGCAGGAGATTCCA
PbAC-R34(NotI)	GCGGCCGCGCCGTGCTCGAAGAACTAGAACCAC
PbAC-R35	CTCCATAGTTTGATATATCCTTTGATTGAC
PbAC-R36	CCATAGTTTGATATATCCTTCAGAAGACTTTCCG
PbAC-R37	CATTCATAAAGCAGCTGTTCCTGTGGCAGG

PbAC-GADF1(NcoI)	GGTACCACCATGGCAAGGAGACAGCGGGAGAAAG
PbAC-GADF2(NcoI)	TCGTCCATGGAAGATGAGCTGAATAACTAC
PbAC-GADR1(BamHI)	TGATTTGGATCCCGTTGATTCCCAAGTCAGC
PbAC-GADR2(BamHI)	CCCCAGGATCCTAAGATGTTTCAAAGAGTTG
PbAC-EF1(NdeI)	GGGATTCATATGGTTAATAGCACAGATCTG
PbAC-EF2(NdeI)	AGTATCCATATGGGACTGTCTCCATTAACTG
PbAC-ER1(XhoI)	CCATGGCTCGAGCGCCGTGCTCGAAGAACT
PbAC-ER2(XhoI)	CTCGCGCTCGAGATTGTGATTAAGATAGTC
PbGPA1	
PbGPA-DGF5	GCYGGWGARYSNGGNAARTCNAC
PbGPA-DGR2	CARTGDATCCAYTTYTTNCK
PbGPA-DGF5R2-3-4-F1	GAACAGCATGCCACTCATAGC
PbGPA-DGF5R2-3-4-R1	TCTAACGGCTCTGTCACTTGG
PbGPA-DGF5R2-3-2-F1	GAGCTTTGCAAGGCTTAACTCG
PbGPA-DGF5R2-3-2-R1	ATCGTGGCTCACGCATTTCGAG
PbGPA1-ExGPAF1	CATATGGGTTGTGGAATGAGC
PbGPA-F1	CGGGATCCGTCATATGGGTTGTGGAATGAGCACC
PbGPA1-F1	TGTCCTTCGCTCCCGTGTTAAGACCACAGG
PbGPA1-F2	TTGCGACTATATCCTCAACCGCTTCGTCTCGCTC
PbGPA1-F3	GACTATATCCTCAACCGCTTCGTCTCGCTCAACC
PbGPA1-F4	GATTGATCGGTTTAAGGAGAAACTGCCTGTTAGC
PbGPA1-F5	CTCTGAGTACGACCAGCTCCTGTTCGAAGACG
PbGPA1-F6	GACATGAAGCAGAACCTGTACATTCCGATATCGTC
PbGPA1-F7	GTGTCATTCATCCATCTTGCTTTACGCC
PbGPA1-F8	GGCTATGGAAGGACTAGATATCCCGTTGGA
PbGPA1-F9	CTGCATGGTTTCCTTCTCAG
PbGPA1-F10	GATCAAAATGCTCTTGCTTGGTGCTGGAGA
PbGPA1-F11	GAATGACTCTGCACGATACTATTTTGACTCCA
PbGPA1-R1	CGGGATCCTCATATCAGTCCACAGAGGCGAAG
PbGPA1-R2	TCCAACGGGATATCTAGTCCTTCCATAGCC or GCTTAGCCTAAACTCTCCATAGTCATCGTGTCC
PbGPA1-R3	GAGCATTTTGATCTCGTTTCTCTGCAGC
PbGPA1-R4	TCCTTGTCCTCGGTGCTCATTCCACACC
PbGPA1-R5	CCTGTGGTCTTAACACGGGAGCGAAGGACA
PbGPA1-R6	TGAAGACTGGCTGGGTTTTGCGGGAAAGGA
PbGPA1-R7	CAATTACCCATGGCTTATTAGG
PbGPA1-R8	CAGTGTATTCGGCACCCCCTTCGTAATCAG
PbGPA1-R9	TGTCCTGGATGATTATATCGTTGACAGCCA
PbGPA1-pYER3	GCCTAGGTCATATCAGTCCACAGAGGCGAAG
PbGPAm-F1	GCTTTTGCTTGGTGCTGTAGAGTCTGGCAAATCCAC
PbGPAm-R1	GTGGATTTGCCAGACTCTACAGCACCAAGCAAAAGC

PbGPA2	
PbGPA2-F1	ACGAACGATACCAAAACCGCGCGGTCATCTACTC
PbGPA2-F2	ACCGCGTCTTTACCCCTGGGTGGTTACCCA
PbGPA2-F3	GGAGATCTACATCCACTACACAAATGCTAC
PbGPA2-F4	TCAATGAGTGCAAACGGGATCTGCAAGGGA or CATGGGTTGCGCAAGTTCGAACGGTGAAGA
PbGPA2-F5	CGCACTGGATGCCACTGGAAGGTGGCTTGA
PbGPA2-F6	CATGGGTTGCGCAAGTTCTCAACCAGTGGA
PbGPA2-F7(EcoRI)	CTAGGAATTCATGGGTTGCGCAAGTTCTCA
PbGPA2-F8	GGTGAAGATTCTTTTGCTTGGTGCTGGAGA
PbGPA2-F9	TTGAGTTTGAAAAGACAAAGCCACTAGCCA
PbGPA2-F10(BD-NdeI)	ATGGCCATGGAGGCCGAACATATGGGTTGC
PbGPA2-R1	TGAAGCATGTCTTGATTGTTGGGTAACCACC
PbGPA2-R2	ATCGAGGAGAATCTTGAATGCAACAACCATG
PbGPA2-R3	GCCGTCGACCACGCGTGCCCTATAGTGA
PbGPA2-R4	CATACGGATCATCTTGTCGATCGCAGACCA
PbGPA2-R5	TGGTGCAGAAACCGAGCGTGGCACAAGAGC
PbGPA2-R6	CTGTTGGCACCTACAGAATCAGGTTGTTGA
PbGPA2-R7	CAATTCCTATCAGAACTCAATCAGCATGCA
PbGPA2-R8	AACGTAGCTCGAGTCCTTCCCCTCTGTTCA
PbGPA2-R9	CTTCGCACTTTTCAAATGCCACGGGACATG
PbGPA2-R10	GGCTTCATGCATTTGATTTGCATTTTGATCC or GAGCACAACTCTCAAGTGAGTGCAACACCA
PbGPA3	
PbGPA3-F1	TCTTGGATTGCGCCAAGGACCTGATAGGAG
PbGPA3-F2	CCTAATGAAGCCGATGTGTTGCGCGCTAGA
PbGPA3-F3	TATCCTCAGTATGTATAGGAAAAGCACACC
PbGPA3-F4	AACCGTGCCGCCAAGTACTTGCTCTGGAGA
PbGPA3-F5	GTATCAGATACCTACCCACCCACAACGT or TGGTATCAGATAGGATGGGTGGGTGTTGCA
PbGPA3-F6	GTGGGTGTTGCAGTTCTGCTTCTGGGGAGA
PbGPA3-F7	CCACTTGGCCTTTTTTCCTTTTCCTTTTCC
PbGPA3-F8	CAGCTCTGGTCCACTTGGCC
PbGPA3-F8(EcoRI)	ATCAGAATTCATGGGTGGGTGTTGCAGTTC
PbGPA3-F9	ATGCAAGATCCTACTGCTTGGTTCGGGTGA
PbGPA3-F10	TGGATTCAGCACCTTACTTCTTCGAGGAAGC
PbGPA3-F11(BD-NdeI)	ATGGCCATGGAGGCCGAACATATGGGTGGG
PbGPA3-R1	TTCACTCCGCTGCCCGCCAACATCGAAC
PbGPA3-R2	ACGCACTGAATACTCAATTGGCCCATTGTG
PbGPA3-R3	GCGTATTCGGGTCCGCGTCAATCTGGTATG
PbGPA3-R4	CCTCTGCTCTGCATTCTCCCCAGAAGCAGA
PbGPA3-R5	CCCTTTCACAAAATACCAGAATCTTTCAGG

PbGPA3-R6	AAAGCAGTGAGTGGCCTGGGGAGTTATACG
PbGPA3-R7	GATTTGATAAGTAAGCGGCCCGATGCGGGA
PbGPA3-R8	GTCGGTGGCTTGAGTAAGGTGAGGATAAAG
PbGPB1	
PbGPB-F1	GCNAARATHTAYGCNATGCAYTGG
PbGPB-F2	TGGGTNATGACNTGYGCNTAYGC
PbGPB-R1	GCRTANGCRCANGTCATNACCC
PbGPB-R2	CCABWKCATRCANGTCATRTCNCC
PbRAS1	
PbRAS-F1(NdeI)	CCGCGCTTCATATGCAGCTTTGTCTAAACC
PbRAS-R1(EcoRI)	GTGATACTGTAGAATTCCACGAAACCCTC
PbTPK1	
PbTPK-F1	CATCCRTTYHTBATTMGAATGTGGGG
PbTPK-F2	TGGGTACTGGHTCYTTTGGNMGNGT
PbTPK-F3	CACGTTCCAGGATGTGAAGAATC
PbTPK-F4	GTTTACTGAGGAAATCGCAGCGCTTC
PbTPK-F5	TGCAAGCCAGTTTGATAGGTATC
PbTPK1-F6	ATCCACCCCTCAACGATGAAGTGG
PbTPK1-F7	GATGCGCCGTATATTCCTCCGGTGAAGGGA
PbTPK1-F8	GACGGAGCCGTATGGGGGTAGTGGGGATGA
PbTPK1-F9	AGACTAACAGCGGGGTCTGTGA
PbTPK1-F10	CTGAGACGGGTCTATGTTCGATGG
PbTPK-F40	GCTGTTTAGTTTACTGAGGAAATCGCAGCGCTTC
PbTPK-R1	ACBTMTTTKGCRAABCCRAARTC
PbTPK-R2	ATSARMAYMCCNARVSWCCACCARTC
PbTPK-R3	CTTCAAATGTCCATATCGATC
PbTPK-R4	CTAAAAGTAGATTCTCTGGCTTTAGGTCTCTG
PbTPK-R5	GTGATGGTCATGTAGATATTCCAACGCCAACG
PbTPK-R6	CTAAAAGTAGATTCTCTGGCTTTAGGTCTCGG
PbTPK1-R7	TACCTGGAACGTGCCCCATAGCG
PbTPK1-R8	TAGGAATGGATGTTTAACCCGCTGCAGC
PbTPK-R9	TAATGTTCTTAATACTGGCGACATTTTGGGAGTG
PbTPK1-R10	ATGTCGCGATTGAACCAGGTGTACTCGC
PbTPK-R11	CTCCGTCTAGATATTGGCATAGGC
PbTPK1-R12	GGGTGAGGTAAACGCAGGGTGCTGGAGCA
PbTPK1-R13	CTTCAGCAAATTGCCTAGACCCCGCATGGA
PbTPK1-R14	GGGACTACGGCAAGGCCTGTGGGTGGATGA
PbTPK1-R15	ATGATGCTGAGGCCCGACGCCACTAGATAG
PbTPK-R16	GTAGAATGGAATTGAACCTGCAGACACTCA
PbTPK1-R17	GTCCACGAAATAATCGCCATATGGATCATCC

PbTPK1-R18	CCCTAAGGGTATCAGGCGTGCCA
PbTPK-R40	CGATCTAAAAGTAGATTCTCTGGCTTTAGGTCTCTG
TPK-DF1	GGNWSNTTYGGNMGNGTNCAYYT
TPK-DF2	CARRTNGARCAYACNAAYGAYGA
TPK-DR1	RTTYTCNGGYTTNARRTCNCKRTA
TPK-DR2	RAACCANGGRTGRTTYTTNAYRTC
TPKL-DF1	CCNTAYGTNGTNTGYDBNTTYGA
TPKL-DF2	GARHTNGAYRTNWSNGTNTAYGA
TPKL-DF3	GANGCNTTYYTNGGNCAYGTNMG
TPKL-DR1	SWCCARAARTCNACCATYTTNGT
PbACTIN	
PbActin-F3	ATGCTCCTCGAGCTGTCTTCCCTTCGATCG
PbActinGADF1	CATATGGAGGAAGAAGTTGCTGCCCTCG
PbActinGADF2	GTGATTCATATGGAGGAAGA
PbActinGADR1	TTAGAAGCACTTGCGATGGACAATAGAGG
PbActin-R3	GGTACATGGTTGTGCCACCGGACATAACGA
PbACTIN-R3(EcoRI)	CGAGGGCGGTGAATTCCTATTGCATACGATC
PbTUPA	
cPbTUPA-F1	ACTTCTTACCTTAGGCCAGCCTCGCCCTCA
cPbTUPA-F2	ACACCCGATGGACGCTGGGTTATGAGTGGA
cPbTUPA-F3	ATCGTGGCGTACAGTTCTGGGATCCAGCGA
cPbTUPA-F4	CCCGTTCAACCCAGCCTACCAACTACCGCA
cPbTUPA-R1	ATTATATGATGACGGCGGTGATAACGAGGA
cPbTUPA-R2	ATAGCTGTAAATCCCATGTCGAGAGTTAGG
cPbTUPA-R3	CCTTCCTCTGGCAAAGTTACGTGTTGCGCA
cPbTUPA-R7	CTGATCATCTCCATCTCCTGGAGTTGGCCA
PbTUPA-F1	MGSACYTTTGARGGNCAYAAR
PbTUPA-R1	CCARATTCKNGCNCKCATRTC
PbTUPA-R2	GTCGCTGGATCCCAGAACTGTACGCCACGA
PbTUPA-R3	CACTCATAACCCAGCGTCCATCGGGTGTCA
PbTUPA-R4	GGATATCCCAGACCTGTAGGTGGA
PbTUPA-R5	GGCAACTTGTCAGGATCCAGGTC
PbTUPA-R6	GAGTGCGGATTTCAGCCTCG
ScGPR1	
ScGPR1-F1	CGGGATCCGAAGTGTGACGAATAAAGC
ScGPR1-F2	GCTGGGACATCAGACTTTCTCTTGCC
ScGPR1-F3	CGGGATCCATATGATAACTGAGGGATTTCCCCCG
ScGPR1-F4	CCGGATCCGTGAAAGTAAAAGAATTAAAGCGC
ScGPR1-F5	CCGGATCCAGGAAAAACCTTGGAACTATTCATG
ScGPR1-R1	CGGGATCCATTTTCAAACATCGCGATAC

ScGPR1-R2	GTTACTAGTTATGTCGCTGTTATCG
ScGPR1-R3	CCGGATCCTTAGATTCTTTTGAATTTGTGCC
ScGPA2	
ScGPA2-F1	CCGGATCCTGGGTCTCTGCGCATCTTCA
ScGPA2-F2	GGGTCTCTGCGCATCTTGAG
ScGPA2-F3	CCGGATCCGTATGGGTCTCTGCGCATCTTCAG
ScGPA2-F4	ATCATGGGTCTCTGCGCATC
ScGPA2-R1	CCGGATCCGCTGTGCATTCATTGTAACAC
ScGPA2-R2	AACGCGAGAAGAGGCATGCAG
Universal primers	
3'AD LD Screening	AAGTGAACTTGCGGGGTTTTTCAGTATCTACGA
5'AD LD Screening	CTATTCGATGATGAAGATACCCCACCAAACCCA
3'AD sequencing	AGATGGTGCACGATGCACAG
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
AP11	GTAATACGACTCACTATAGGGCACGC
AP21	TCACTATAGGGCACGCGTGGTCGAC
AP3	TACGACTCACTATAGGGCACGCGTGGTCGA
AP4	GTAATACGACTCACTATAGGGCACGCGTGG
3'DNA-BD Screening	TGGCTGCAAGCGCGCAAAAAACCCCTCAAGAC
5'DNA-BD Screening	TCATCGGAAGAGAGTAGTAACAAAGGTCAAAGA
3'BD sequencing	CGCAAAAAACCCCTCAAGAC
CDS/3'BamHI(2)	ATTCTAGACGCGGATCCGCGGACATGTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTVX
CDS/3'BamHI(3)	GATTCTAGACCGGATCCGCGGACATGTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTVN
CDSIII/3'BamHI	AACGCGGATCCGCGTTTTTTTTTTTTTTTTTTTTTTTT TTTTT
Creator-F1	CGACGGTACCGGACATATGCCCGGGAATTC
Creator-R1	GCGCGCCAAACGAATGGTCTAGAAAGCTTC
CYC1 Reverse (pYES6)	GGAGGGCGTGAATGTAAGCGTGAC
GADF1	GAAGAGAAAGGTCGAATTGGGTACCG
GADR1	CGCGGATCCGTATCGATGCCCACCCGGGTGGAATTC
GADR2	CGCGGATCCGTGTATCGATGCCCACCCGGGTGGAATTC
GAL1 Forward (pYES6)	CCTCTATACTTTAACGTCAAGGAG
5'LONG	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCA ACGCAGAGT
3'LONG	CTAATACGACTCACTATAGGGCATTCTAGAGGCCGA GGCGGCCGA
M13F1	ACCGCCTTACGCGTGTAAAACGACGGCCAG
M13 forward	GCGTGTAAAACGACGGCCAG

M13R1	CCAGGATCTCCTAGGGAAACAGCTATGACC
M13 reverse	GGGAAACAGCTATGACCATG
3-2PB	AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTT TTTTTTTTTTTTTTTTTVN
5-2PB	AAGCAGTGGTATCAACGCAGAGTACGCGGG
pGEMTE-SP6	AGGTGACACTATAGAATACTCAAG
PolyT	TTTTTTTTTTTTTTTTTTTTTTTTVN
pQE-F1	TGTGAGCGGATAACAATTTCACACAG
pQE-R1	CAACCGAGCGTTCTGAACAAATCCAG
5'RACE	AAGCAGTGGTATCAACGCAGAGT
3'RACE	ATTCTAGAGGCCGAGGCGGCCGACAT
SHORT	CTAATACGACTCACTATAGGGC
SIV1	AAGAAGTGGTATCAACGCAGAGTGGCCATTACG
SIV2	GTATCAACGCAGAGTGGCCATTACGGCCGG
SIVBamHI	AAGCAGTGGTATCAACGCAGAGTGGATCCGGG
Smart IV	AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCC GGG
T3	AATTAACCCTCACTAAAGGG
T7	TAATACGACTCACTATAGGG
T7 Sequencing	TAATACGACTCACTATAGGGC
V5C	ACCGAGGAGAGGGTTAGGGAT

2.6.2 Routine PCR

PCR was routinely carried out according to the manual of the product providers. A certain number of PCR reactions were performed with adjusted conditions; for example, increased primer concentration for PCR with degenerate primers. The annealing temperature and number of cycles could vary greatly according to the melting temperature of primers and the abundance of target gene in template samples. Here, a typical reaction composition is given below, when using HotStarTaq PCR system.

Reaction composition	Final concentration
10X PCR Buffer	1X
dNTP (10 mM each)	200 μM
Primer A (50 μM)	0.5-1 μM

Primer B (50 μM)	0.5-1 μM
HotStarTaq Polymerase (5 u/μl)	2.5 u/reaction
Template DNA	Up to 1 μg/reaction
H2O	To 50 μl

Cycling parameters are as below.

1 cycle	95°C	15 minutes
30 cycles	94°C	30 seconds
	50-65°C	30 seconds
	72°C	1-5 minutes
1 cycle	72°C	6 minutes

PCR reactions were performed in an Eppendorf Gradient Thermocycler. The lid of the machine was set to 105°C, removing the need for mineral oil overlay in PCR tubes.

2.6.3 Single Specific Primer PCR (SSP-PCR)

SSP-PCR was used to isolate gene fragments when the full-length gene sequence was unknown but limited sequence information was available. This technique has been used extensively in this thesis to clone genes of *Pb01* from 2 cDNA libraries and a universal GenomeWalker library. For cDNA libraries, *Pb01* cDNAs were cloned into λ-ZAPII phage vector and pDNR-LIB plasmid vector. For construction of the GenomeWalker library, *Pb01* genomic DNA was digested with blunt cutting restriction enzymes followed by universal adaptor ligation at both ends. SSP-PCR was done with a gene specific primer and a standard universal primer that annealed either at the boundary of the vector and the cDNA, or at the universal adaptor. In most situations, a two-step nested SSP-PCR was applied when no obvious specific band was observed with a single-step amplification.

2.6.4 RT-PCR and Nested 5' SMART RACE RT-PCR

RT-PCR was performed in two steps, i.e. RT followed by PCR. RT was undertaken using Clontech's PowerScript Reverse Transcriptase according to its manual. Usually, 0.5-1 μg *Pb01* mRNA was used as template in a 20-μl RT reaction. The RT product was directly used as the template for PCR with 1 μl RT product in each 50-μl volume of PCR reaction. In order to determine the transcriptional start site of a cDNA sequence, nested 5' SMART RACE RT-PCR was applied. The SMART (Switching Mechanism At 5' end of RNA Transcript) technique is facilitated by paring the 3' end of the first strand cDNA with a 5' adaptor (Smart IV oligonucleotide from Clontech) which in turn serves as the second template for RT to switch to and extend first-strand synthesis to the end of the adaptor. The PowerScript Reverse Transcriptase possesses the activity of adding 3 to 5 extra cytosine nucleotides to the 3' end of the completed first cDNA strand. This poly-C motif allows the annealing of the 3' end GGG sequence of the Smart IV oligo. Primers derived from Smart IV oligo, coupled with a reverse gene specific oligo, allow 5' RACE (Rapid Amplification of cDNA End). Usually, nested secondary PCR is necessary because it was hard to produce a sharp specific band after primary amplification. The typical reaction components and conditions are presented as below:

RT

Pb01 mRNA	1 μl
Reverse gene specific primer (2 μM)	1 μl
Smart IV oligo (10 μM)	2 μl
H2O	7 μl

The mixture was heated to 70°C for 2 minutes and cooled on ice. Then the following components were added:

5X First-Strand Buffer	4 µl
dNTP (10 mM each)	2 µl
DTT (100mM)	2 µl
PowerScript RT	1 µl

The mixture was incubated at 42°C for 1 hour. Reaction was terminated by heating at 70°C for 15 minutes.

Primary PCR

PCR was performed using the HotStarTaq PCR kit.

RT Product	1 µl
10X Buffer	5 µl
dNTP (10 mM)	1 µl
SIV1 (50 µM)	1 µl
Reverse gene specific primer (50 µM)	2 µl
HotStarTaq	0.5 µl
H2O	To 50 µl

Cycling parameters used are as below:

1 cycle	95°C	15 minutes
31 cycles	94°C	30 seconds
	68°C	30 seconds
	72°C	3 minutes
1 cycle	72°C	10 minutes

Secondary PCR

A secondary PCR was performed using SIV2 and a nested gene specific primer. About 0.1 µl of the primary PCR reaction was used as a template. The cycling parameters in the primary PCR were repeated, except that the annealing temperature was changed to 65°C.

2.6.5 A-Tailing of PCR Products

When hi-fidelity cDNA or gene fragments were amplified with ProofStart Taq polymerase, blunt ended PCR products were produced and an A-tailing procedure was required to facilitate cloning the PCR products into pGEM-T Easy vector. The procedure is outlined below:

PCR fragment	5 µl
10X Buffer (Promega)	1 µl
dATP (10 mM)	0.2 µl
Taq DNA Polymerase in Buffer B (Promega)	1 µl
H2O	To 10 µl

The mixture was incubated at 70°C for 15-30 minutes. After cooling on ice, 2 µl of the mixture was used in a ligation reaction with pGEM-T Easy vector (Promega).

2.7 Cloning Techniques

Plasmid extraction was usually done with Qiagen's Miniprep kit. When a large amount of plasmid was required for preparation of libraries, Qiagen's plasmid Maxi kit was used. Restriction enzyme digestion of DNA was performed mainly using Promega's system. For cloning purpose, plasmid or DNA fragment elution from plasmid miniprep, gel extraction, or PCR purification was directly subjected to

digestion. Digestions usually proceeded for 3-4 hours at 37°C and terminated by heating at 65°C for 15 minutes. Digested vectors and insert fragments were recovered using Qiagen's Gel Extraction kit. PCR fragment purification was performed using Qiagen's Gel Extraction kit or PCR Purification Kit. Quantification of DNA was done by comparing the intensity of fluorescence from the target DNA band with those of Low Mass DNA Ladder (Invitrogen) bands after eletrophoresis. When single restriction digestion was applied in cloning, dephosphorylation of both ends of the digested vector was needed to reduce the frequency of self ligation. Shrimp AP (alkaline phosphatase, Roche Biochemicals) was used to this end. After dephosphorylation, the reaction was heated to inactivate the AP. The treated vector was directly used in ligation without further purification. Ligation reactions were carried out with Pormega's T4 DNA ligase, using a 1:3 molar ratio of vector to insert, and incubating the reaction mix at 4°C for overnight. Transformation of plasmid into *E. coli* was carried out using commercial competent cells NovaBlue (Novagen) and DH5α (Invitrogen) and XL10-Gold (Stratagene). Competent cells were prepared according to the calcium chloride treatment protocol described previously (Sambrook et al, 1989). When transformation was performed, 50 μl of competent cells mixed with 2 μl of ligation mixture was incubated on ice for 5 minutes, heat-shocked at 42°C for 30 seconds and snap cooled on ice for 2 minutes. Cells were then directly plated out if ampicillin or carbenicillin was used for selection. For selection with kanamycin resistance, cells were made more robust by shaking in SOC medium (Novagen) at 37°C for 1 hour before plating. For blue/white screening in TA cloning, IPTG/X-Gal were mixed with cells and spread on LB-ampicillin plates. Positive clones on selective plates were confirmed by restriction digestion of extracted plasmid. Sequencing was used to re-confirm the

accuracy of cloning if the constructions were important enough for downstream work. Sequencing is another interesting aspect of this thesis in that at least 100 kb of DNA has been sequenced based on a conservative estimation. Sequencing reactions were performed by BaseClear (Netherlands) and University of Durham. The data were analyzed using VectorNTI software (Informax).

2.8 DNA Extraction

Pb01 DNA and *S. cerevisiae* AD1-8^{-} DNA were extracted using Qiagen's DNeasy Plant Maxi kit. The purification was done by introducing a small modification into the kit manual. Materials provided from the kit appear underlined in the following procedures.

1. *Pb01* yeast form cells were grown in modified YPD for 3 days at 37°C. *S. cerevisiae* cells were obtained by overnight culture in YPD at 30°C.

2. Fungal cells were precipitated by centrifugation at 4,200 rpm for 10 minutes. About 1 gram of wet cells was used for each Qiagen's Maxi column. Cells were ground into powder in liquid nitrogen in a mortar. The powder was then transferred to a 50-ml tube containing 5 ml of Buffer AP1. 10 µl RNase A was added in each tube and the mixture was incubated for 10 minutes at 65°C.

3. 1.8 ml of Buffer AP2 was added to the lysate and the mixture was incubated for 10 minutes on ice followed by centrifugation at 4,200 rpm for 5 minutes at room temperature.

4. The supernatant was decanted to the QIAshredder column. After spinning at 4,200 rpm for 5 minutes, the flow-through was transferred to a new 50-ml tube and the volume was recorded.

5. 1.5 volumes of Buffer AP3/E was added to the clear lysate and the mixture was applied to a DNeasy Maxi spin column. The column was centrifuged at 4,200 rpm for 5 minutes and washed with 12 ml Buffer AW.

6. Genomic DNA was eluted with hot (65°C) 1.5 ml Buffer AE. DNA was quantified by measuring the optical density of a diluted sample at a wavelength of 260 nm. About 1 μg of DNA was run in a 1% agarose gel for quality control.

2.9 Total RNA and mRNA Extraction

Pb01 total RNA and mRNA were obtained using Qiagen's RNeasy Midi kit and Oligotex mRNA Midi kit respectively. Materials provided from the kit are underlined.

Total RNA extraction

1. *Pb01* yeast cells were grown in 50 ml modified YPD with shaking at 110 rpm for 4 days to obtain log phase cells. Cells were precipitated and re-suspended in 50 ml of fresh modified-YPD medium. The cells were shaken at 160 rpm for an additional 6 hours at 37°C. Cells were again harvested and re-suspended in 10 volumes of RNAlater (Ambion) and stored at -80°C.

2. On the day of RNA extraction, RNAlater was discarded by centrifugation and cells

were broken in liquid nitrogen. About 1 gram of cell powder was transferred to a 50-ml tube containing 10 ml Buffer RTL to make the cell lysate.

3. The cell lysate was centrifuged for 10 minutes at 4,200 rpm. The supernatant was saved and mixed with 0.5 volume of 100% ethanol.

4. The sample was passed through the RNeasy Midi column by centrifugation. The column was washed by Buffer RW1 once and Buffer RPE twice.

5. For each column, 250 µl RNase-free water was used to elute RNA.

6. The total RNA was quantified by measuring the optical density of a diluted RNA solution at a wavelength of 260 nm as well as qualified by running 5 µl of the total RNA on a 1% agarose gel followed by ethidium bromide staining.

mRNA Extraction

1. For each 250 µg of *Pb01* total RNA, the volume of the sample was adjusted to 500 µl with RNase-free water, then 500 µl Buffer OBB and 20 µl Oligotex Suspension were added.

2. The sample was heated at 70°C for 3 minutes to disrupt secondary structure.

3. The sample was then placed at room temperature for 10 minutes to allow hybridization between the oligo-dT30 and the poly-A tail of the mRNA.

4. The Oligotex:mRNA complex was precipitated by centrifugation, re-suspended in 400 µl Buffer OW2 and applied onto a small spin column. This was washed by passing Buffer OW2 through the column twice with centrifugation.

5. Pb01 mRNA was eluted with 100 µl hot (70°C) Buffer OEB.

6. The mRNA was quantified by measuring the optical density of a diluted mRNA solution at a wavelength of 260 nm. The quality of the mRNA was tested by running 1 µg of mRNA on a 1% agarose gel and ethidium bromide staining.

2.10 Construction of λ-ZAP-cDNA Library of *Pb01*

The λ-ZAP-cDNA expression library was constructed by using a ZAP-cDNA Synthesis Kit and a ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene) with modifications to the instruction manual. First strand synthesis of cDNA was primed with an oligo(dT) linker-primer which contained an *Xho*I site and was transcribed using MMLV-RT and 5-methyl dCTP, which protected the synthesized cDNA from *Xho*I digestion. Second strand synthesis was nick-translated using a nucleotide mixture supplemented with dCTP to ensure that the *Xho*I site in the linker-primer was digested. The uneven termini of the double-stranded cDNA were blunted with *Pfu* DNA polymerase and *EcoR*I adapters ligated to the blunt ends. *Xho*I digestion released *Xho*I and *EcoR*I cohesive ends to allow the cDNA to be inserted into the Uni-ZAP XR vector in a sense orientation (*EcoR*I-*Xho*I) with respect to the *lacZ* promotor. The lambda library was packaged using Gigapack III Gold Packaging Extract and amplified by plating on *E. coli* cell line XL1-Blue MRF'. XL1-Blue MRF', an McrA$^-$McrB$^-$ *E. coli* strain, was used as host for Uni-ZAP-hemimethylated

cDNA phage because hemimethylated DNA introduced into an $McrA^+McrB^+$ strain would be subject to digestion by the *mcrA* and *mcrB* restriction systems. After passing through XL1-Blue MRF' cells, the cDNA library was no longer hemimethylated and could be grown in $McrA^+McrB^+$ strains such as XL1-Blue. The procedure for cDNA synthesis, packaging and amplification of the λ-ZAP-cDNA is briefly summarized as follows. The materials provided in the kits or prepared according to the instruction are underlined.

1. First-strand synthesis. The following components were added into an Eppendorf tube in order:

4.5 μl of 10×first-strand buffer

3.0 μl of first-strand methyl nucleotide mixture

2.0 μl of linker-primer (1.4 μg/μl)

31 μl of mRNA (7 μg)

1.0 μl of RNase Blocking Ribonuclease Inhibitor (40U/μl)

3.5 μl of MMLV-RT (20 U/ μl)

First-strand reactions were incubated at 42°C for 1 hour.

2. Second-strand synthesis. The 45 μl of first-strand reaction mix was placed on ice and supplemented with the following reagents in order:

20.0 μl of 10×second-strand buffer

6.0 μl of second-strand nucleotide mixture

116 μl of sterile distilled water

2 μl of RNase H (1.5 U/μl)

11 μl of DNA polymerase I (9 U/μl)

The contents were mixed and incubated at 16°C for 2.5 hours.

3. Blunt-ending the cDNA termini and ligation with *EcoR*I adapters. After the second-strand synthesis, the tube was placed on ice and then the following were added:

23 μl of blunting dNTP mix

2.0 μl of cloned *Pfu* DNA polymerase

The tube was incubated at 72°C for exactly 30 minutes, then was extracted with phenol-chloroform and precipitated with sodium acetate and ethanol. After overnight precipitation, the blunt-ended cDNA was pelleted and dried. The pellet was then resuspended in 9.0 μl of *EcoR*I adapters. The following components were added to the tube containing the blunt-ended cDNA and the *EcoR*I adapters:

1.0 μl of 10×ligase buffer

1.0 μl of 10 mM rATP

1.0 μl of T4 DNA ligase (4 U/μl)

The ligation was incubated at 4°C for 2 days.

4. Phosphorylation of the *EcoR*I ends and *Xho*I digestion. The ligation mix was inactivated by heating the tube at 70°C for 30 minutes and cooling it down to room temperature. The adaptors were phosphorylated by adding the following and incubating at 37°C for 30 minutes.

1.0 μl of 10×ligation buffer

2.0 μl of 10 mM rATP

6.0 μl of sterile water

1 μl of T4 polynucleotide kinase (10 U/μl)

The kinase was heat inactivated at 70°C for 30 minutes. *Xho*I digestion was done by adding the following components to the kinase tube and incubated for 1.5 hours at 37°C:

28.0 μl of *Xho*I buffer supplement

3.0 μl of *Xho*I (40U/μl)

5. Size fractionation. Fractionation of large size cDNA increases the quality of the cDNA library. 50-μl of a *Xho*I digest was mixed with 50 μl H_2O and 2 μl 1% xylene cyanol. The mixture was run through Clontech's CHROMA SPIN-400 column according to the instruction from the manufacturer. The passage of the first 250 μl, corresponding to the cDNA with sizes larger than 500 bp, was collected and extracted with phenol-chloroform. The cDNA was then precipitated with sodium acetate and ethanol, and re-suspended in 5 μl of sterile water. The concentration of the cDNA was determined to be 40-50 ng/μl by an ethidium bromide plate assay.

6. Ligating the cDNA into the Uni-ZAP XR vector arms. The ligation was set up as the following and incubated at 12°C overnight:

2.5 μl of resuspended cDNA (about 100 ng)

0.5 μl of 10×ligase buffer

0.5 μl of 10 mM rATP

1.0 μl of the Uni-ZAP XR vector (1 μg/μl)

0.5 μl of T4 DNA ligase (4 U/ μl)

7. Packaging. 2.5 μl of ligated Uni-ZAP-cDNAs were added to 20 μl of packaging extract. The tube was incubated at room temperature for 2 hours. After adding 500 μl of SM Buffer and 20 μl of chloroform, the tube was spun briefly and the supernatant was saved and stored at 4°C.

8. Plating and titrating. 2 μl of the packaged ligation were serially diluted 10 fold. 1 μl of each dilution was used in plating and titrating by the following mixing procedure:

1 μl of a dilution

200 μl of XL1-Blue MRF' cells at OD_{600} of 0.5

The mixtures were incubated at 37°C for 15 minutes to allow the phage to attach to the cells and the following components were added to each tube of cells:

3 ml of NZY top agar (48°C)

15 μl of 0.5 M IPTG

50 μl of X-gal (20 mg/ml)

The mixtures were immediately plated onto NZY agar plates and incubated at 37°C overnight. The next morning, approximately 75% of plaques were observed to be white, and the whole packaging reaction contained 40,000 plaque forming units.

9. Testing the average size of cDNA insertions. The average size of the cDNA library was determined by PCR amplification of individual plaques with T3 and T7 primers. The PCR products were run on a 1% agarose gel and stained with ethidium bromide.

10. Amplification of the λ-Uni-ZAP-cDNA library. Aliquots containing 10,000 plaque forming unites (pfu) of the library were mixed with 600 µl of XL1-Blue MRF' cells, which were grown in LB supplemented with 10 mM MgSO4 and 0.2% maltose to an OD_{600} of 0.5. The mix was incubated at 37°C for 15 minutes, then mixed with 6.5 ml of NZY top agarose and spread evenly onto 150-mm plates of NZY agar. The plates were then incubated at 37°C for 6-8 hours until the plaques grew to 1-2 mm. Then the plates were overlaid with 10 ml of SM buffer and gently shaken at 4°C overnight. The next morning, the bacteriophage suspension was collected and mixed with a 1/20 volume of chloroform. Cell debris and chloroform were removed by centrifugation. The supernatant was mixed with dimethylsulfoxide (DMSO) to a final concentration of 7%, then aliquoted and stored at -80°C. The *P. brasiliensis* cDNA library thus constructed, amplified and titrated similarly to the procedure mentioned above; indicating a titer of 3×10^8 pfu/ml.

2.11 Creator SMART cDNA Library Construction of *Pb01*

Clontech's Creator SMART cDNA Library Construction Kit is the combination of the Creator technology and SMART technology. The cDNAs are cloned into pDNR-LIB Creator vector which serves as donor vector. The Creator system allows the cloned cDNAs to be transferred directly into multiple acceptor expression vectors using Cre-*loxP* recombination without the need for sub-cloning. However, in this thesis, the Creator function has not been used. The SMART (Switching Mechanism At 5' end of RNA Transcript) is used in cDNA synthesis to enrich full-length cDNAs and eliminate adaptor ligation. The mechanism of SMART has already been explained in section 2.6.4 of this chapter. In general, cDNA synthesized with the kit contains the complete 5' end of the mRNA, as well as the sequence complementary

to the SMART IV oligo, which then serves as a universal priming site (SMART anchor) in downstream applications. The construction procedure of pDNR-*Pb01*cDNA Library is described below. The materials provided in the kit are underlined.

1. First-strand cDNA synthesis. The following reagents were combined in a 0.5-ml RNase-free tube.

2 µl of mRNA sample (1 µg mRNA)

1 µl of SMART IV oligonucleotide

1 µl of CDSIII/3' PCR Primer

Deionized H_2O was added to make a total volume of 5 µl. The tube was incubated at 72°C for 2 minutes then cooled on ice for 2 minutes. The following were added:

2 µl of 5X First-Strand Buffer

1 µl of DTT (20mM)

1 µl of dNTP Mix (10 mM)

1 µl of PowerScript Reverse Transcriptase

The contents were mixed and the tube was incubated at 42°C for 1 hour in an air incubator. The reaction was terminated by adding 1 µl of Sodium Hydroxide and incubating at 68°C for 30 minutes.

2. Double-strand synthesis by primer extension. The following components were combined:

11 µl of First-strand cDNA

71 µl of Deionized H_2O

10 µl of 10X Advantage 2 PCR Buffer

2 µl of 50X dNTP Mix

2 µl of 5' PCR Primer

2 µl of CDSIII/3' PCR Primer

2 µl of 50X Advantage 2 Polymerase Mix

The primer extension was performed using the following program:

95°C for 1 minute

72°C for 10 minutes

3 cycles of 95°C for 20 seconds and 68°C for 8 minutes

3. Proteinase K digestion. 2 µl of proteinase K was added to 50 µl of amplified double-strand cDNA. The tube was incubated at 45°C for 20 minutes. Proteinase K was then eliminated by phenol:chloroform extraction. DNA was precipitated with sodium acetate. The DNA pellet was re-suspended with 79 µl deionized H_2O.

4. *Sfi* digestion. The amplified DNA was digested with the following components:

79 µl of cDNA

10 µl of 10X *Sfi* Buffer

10 µl of *Sfi* Enzyme

1 µl of 100X BSA

The digestion was incubated at 50°C for 2 hours. Then 2 µl of 1% xylene cyanol was added to the tube.

5. cDNA size fractionation. The cDNAs were fractionated using a CHROMA SPIN-400 column. Single-drop fractions were collected and the first 3 drops were the peak fractions collected. The fractionated cDNA was precipitated with sodium acetate

and re-suspended in 7 μl of deionized H_2O.

6. Ligation of double-strand cDNA into the pDNR-LIB vector. Ligation was performed by mixing the following components.

1 μl of cDNA

1 μl of pDNR-LIB

0.5 μl of 10X Ligation Buffer

0.5 μl of 10 mM ATP

0.5 μl of T4 DNA Ligase

1.5 μl of deionized H_2O

The ligation was incubated at 16°C overnight.

7. Transformation. The ligation mixture was transformed into XL10-Gold Kan^r Ultracompetent Cell (Stratagene) and plated out on LB-Chloramphenicol (30 μg/ml) plates. About 1X 10^6 transformants were obtained. To test the average size of inserts for quality control, 30 colonies were selected and subjected to PCR with M13 Forward and M13 Reverse primers. The library construction was finished by extracting library plasmids with Qiagen's Plamid Maxi Kit. The concentration of library plasmids was determined by measuring their optical density at 260 nm.

2.12 Screening the cDNA Library with a Non-radio-labelled DNA Probe

To clone the *Pb* adenylate cyclase gene (*PbCYR1*), the λZAPII-cDNA library was screened. Initially, a specific fragment (389-bp) of *PbCYR1* was obtained by PCR with 2 degenerate primers, i.e. PbAC-F1 and PbAC-R1. This fragment was used for labelling and screening the λZAPII-cDNA library. The experiments were based on

Amersham's Gene Images Random Prime Labelling Kit and Gene Images CDP-Star Detection Kit, in combination with Stratagene's ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit. The procedures for the experiments are detailed as below. Materials from the kits as well as prepared according to product manuals are underlined.

Preparation of labelled probe

About 50 ng of DNA fragment was used for one labelling reaction. The DNA sample was denatured for 5 minutes in boiling water and then snap cooled on ice. The following reaction mix was incubated at 37°C for 1 hour:

9 µl of H_2O

25 µl of denatured DNA

10 µl of Nuceotide Mix

5 µl of Random Primers

1 µl of Klenow Enzyme Solution (5 U/ µl)

The reaction was terminated with EDTA and stored at -20°C before use.

Plating, lifting and cross-linking

The λZAPII-cDNA library phage was diluted with SM buffer. About 6,000 pfu were incubated with 0.6 ml of XL1-Blue MRF' cells (OD_{600}=1) and plated out after mixing with NZY top agar. Plates were incubated at 37°C overnight to allow phage growth. A Hybond-N+ (Amersham) membrane was cut and marked with a ball point pen, and then overlaid on a pre-cooled phage plate. The orientation was assigned by pricking with a needle on the overlaying membrane and the agar underneath. The membrane was lifted after 5 minutes. Phage DNA attached to the membrane was

denatured with denature solution, neutralized with neutralization solution and rinsed with rinse buffer. DNA was cross-linked to the membrane with Stratalinker 1800 for 1 minute. The membrane was stored at -20°C before hybridization.

Hybridization

The hybridization buffer was prepared according to the following formula.

5X SSC (prepared from 20X SSC)

0.1% SDS (prepared from 10% SDS)

5% dextran sulphate

20 fold diluted liquid block

The hybridization buffer was preheated to 60°C prior to membrane emersion. Pre-hybridization was carried out for 2 hours with agitation in an Amersham's Hybridization Oven. The labelled probe was denatured by boiling for 5 minutes and snap cooled on ice. The probe was then added into the pre-hybridization solution and hybridization was performed overnight at 60°C. Stringency washes were done at 60°C with 1X SSC plus 0.1% SDS once, then twice with 0.5X SSC plus 0.1% SDS. Frequently, the membrane was stored at 4°C before image detection.

Image detection

After hybridization, the membrane was blocked with liquid blocking (10 fold diluted in Buffer A) at room temperature for 1 hour. The membrane was incubated with anti-fluorescein-AP (5,000 fold dilution in 0.5% BSA plus Buffer A) for 1 hour followed by 3 washes with 0.3% Tween 20 in Buffer A. For image detection, the membrane was soaked in detection reagent for 2 minutes, wrapped in a plastic sheet and exposed to an X-ray film.

The second and third screening

After image detection, phage plaques causing positive images on an X-ray film were cored and soaked in SM buffer. PCR was done to confirm that the phage solution contained the target gene. About 1,000 pfu were plated out for the secondary screening and 100 pfu for the third screening. Pure phage plaques containing the target gene were obtained after the third screening.

Single-clone excision

The principle of the *in vivo* excision of the pBluescript phagemid from the Uni-ZAP XR vector is described in the instruction manual of the ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene). The purified plaques were converted into pBSK plasmid. The following components were combined in a 50-ml tube and incubated at 37°C for 15 minutes:

200 μl of freshly grown XL1-Blue MRF' cells at an OD_{600} of 1.0

250 μl of purified plaques ($>1\times10^5$ phage particles)

1 μl of the ExAssist helper phage ($>1\times10^6$ pfu/μl)

3 ml of LB broth were added to the incubation and again incubated at 37°C for 3 hours. The tube was then heated at 70°C for 15 minutes and centrifuged at 4,000×g for 15 minutes. The supernatant containing filamentous phage particles of excised pBluescript phagemid was used to transfect fresh SOLR cells by the following combination and incubation at 37°C for 15 minutes:

100 μl of the phagemid supernatant

200 μl of SOLR cells at OD_{600} of 1.0

50 μl of the transfection reaction was plated on LB-ampicillin agar plates (50 μg/ml)

and incubated overnight at 37°C. Colonies appearing on the plates contained the pBSK plasmids with the target gene. The plasmids were purified with Qiagen's plasmid MiniPrep kit.

2.13 Universal GenomeWalker SSP-PCR

Genomic walking was used to obtain DNA sequence information on *Pb01* genes. Clontech's GenomeWalker procedure is a simple method for walking upstream or downstream of a genomic DNA from a known sequence such as a cDNA or a DNA fragment obtained by PCR with degenerate primers. The GenomeWalker Universal Kit was used to construct 4 *Pb01* GenomeWalker libraries. High quality *Pb01* DNA was digested with 4 blunt cutting restriction enzymes (*Dra*I, *Eco*RV, *Pvu*I and *Stu*I). Each batch of digested genomic DNA was then ligated separately to the GenomeWalker Adaptor, which serves as an anchor in subsequent SSP-PCR amplifications. SSP-PCR was used to acquire new DNA sequence information with ABgene's Extensor Hi-Fidelity PCR Enzyme Mix. Usually, 2 PCR amplifications were necessary if no specific single band was observed with an one-step amplification. Specific bands obtained from different libraries were recovered by gel extraction and subjected to DNA sequencing. The detailed protocols for library constructions and SSP-PCR are given below. Materials from the kit are underlined.

Digestion of genomic DNA

The quality of *Pb01* genomic DNA was checked on a 0.5% agarose gel. A single band was observed without obvious smearing. The DNA was subjected to 4 restriction digestions as mentioned above. The following components were combined:

25 µl of genomic DNA (100 ng/µl)

10 µl of 10X Restriction enzyme buffer

10 µl of Restriction enzyme (10 U/µl)

57 µl of Deionized H_2O

The reactions were incubated at 37°C for 2 hours, then briefly vortexed and incubated at 37°C for a further 18 hours. After incubation, the efficiency of digestions was assessed by running 5 µl of each reaction on a 0.5% agarose gel. The remaining 95 µl of digested DNA was purified by phenol extraction and concentrated by precipitation with sodium acetate and ethanol. The pellet was dissolved in 20 µl of TE buffer (pH7.5).

Ligation of GenomeWalker Adaptors

For each library construction, the following components were added:

4 µl of digested DNA

1.9 µl of GenomeWalker Adaptor

1.6 µl of 10X Ligation buffer

0.5 µl of T4 DNA ligase (6 units/µl)

The reaction was incubated at 16°C overnight followed by termination at 70°C for 5 minutes. For each construction, 72 µl of TE (pH7.5) was added before application in SSP-PCR.

Genome walking by using SSP-PCR

SSP-PCR was performed with ABgene's Extensor Hi-Fidelity PCR Enzyme Mix, which contains a combination of 2 DNA polymerases suitable for long distance PCR. Usually a two-step nested PCR was applied to achieve high specificity of

amplification. Therefore, 2 pairs of primers were employed, with each pair composed of a gene specific primer and an anchor primer derived from the adaptor sequence. SSP-PCR was done as follows with materials from the kit underlined:

Primary PCR

To a thin-walled 0.5 ml PCR tube was added the following.

1 µl of GemoneWalker library DNA

5 µl of 10X Extensor Buffer

1 µl of dNTP (10 mM each)

1 µl of gene specific primer (50 µM)

1 µl of AP1 or AP11 or AP3 (10 µM)

0.5 µl of Extensor Hi-Fidelity PCR Enzyme Mix (5U/ µl)

To 50 µl of Dionized H_2O

Cycling parameters are given below:

1 cycle	94°C	2 minutes
5 cycles	94°C	10 seconds
	68°C	30 seconds
	68°C	3 minutes
5 cycles	94°C	10 seconds
	65°C	30 seconds
	68°C	3 minutes
25 cycles	94°C	10 seconds
	60°C	30 seconds
	68°C	3 minutes
1 cycle	68°C	7 minutes

For each primary PCR, 5 µl of the product was analyzed on an agarose gel.

Secondary PCR

The primary PCR product was 1:50 diluted in H_2O before serving as the template for the secondary PCR. PCR conditions were the same as those used in the primary PCR except that the final concentration of the anchor primer (AP2, AP21 or AP4) was increased to 1 μM. Also the 5 cycles with an annealing temperature of 68°C were deleted.

2.14 Construction of Random Mutagenesis Libraries for Yeast Two-Hybrid Screening

In order to find potential protein interactions, genes of the cAMP signaling pathway were randomly mutated, cloned into pGADT7 prey vector and subjected to yeast two-hybrid screening. The screening proved to be essential in some situations because mutations can lead to a change of protein activity or the interacting domain can only be exposed when one part of the protein is removed. Stratagene's GeneMorph II Random Mutagenesis Kit was used for random mutagenesis. A novel error prone PCR enzyme blend, Mutazyme II, is used in this kit to generate mutated PCR products. The advantage of Mutazyme II is the production of a less biased mutational spectrum with equivalent mutation rates for all the 4 nucleotides. Therefore, libraries created with Mutazyme II should exhibit greater mutant representation compared to libraries generated with other enzymes. In addition, the mutation frequency can be controlled by adjusting the initial target DNA amounts and the number of cycles. In this thesis, PCR parameters were selected to create a mutation frequency of 0-4.5 mutations/kb because 1 amino acid change per gene was desired. The procedure for library constructions is described as below with materials

from the kit underlined.

Random mutagenesis PCR components

5 μl of 10X Mutazyme II reaction buffer

1 μl of 40 mM dNTP

X μl of template (100 ng target gene, not the whole plasmid)

0.5 μl of forward primer (50 μM)

0.5 μl of reverse primer (50 μM)

1 μl of Mutazyme II DNA polymerase (2.5 U/μl)

To 50 μl of H_2O

Cycling parameters

1 cycle	95°C	2 minutes
18, 20, 22, 24 cycles	95°C	30 seconds
(four tubes)	60°C	30 seconds
	72°C	1 minute
1 cycle	72°C	10 minutes

The 4 tubes of reactants, which had undergone 18, 20, 22 and 24 cycles respectively, were mixed and the amplified band was purified by gel extraction. The mutated DNA was double digested with suitable enzymes and ligated into the pGADT7 prey vector. The ligation was transformed into XL10-Gold Ultracompetent Cells to generate over 2 X 10^4 transformants. Randomly selected clones were subjected to sequencing to check the resultant mutations. Library plasmids were extracted with Qiagen's plasmid Maxi columns or MiniPrep columns.

2.15 Yeast Two-Hybrid

Clontech's Matchmaker Yeast Two-Hybrid System 3 was used to analyze protein interactions with the *Pb01* cAMP signaling pathway. It is a sensitive method for detecting relatively weak and transient protein interactions. Because the protein interaction is performed *in vivo*, the proteins are supposed to be in their native form, which leads to increased sensitivity and accuracy. The system is specific as well because a new GAL4-based two-hybrid system is featured to reduce false positives. In general, a bait gene is cloned into the vector pGBKT7 and expressed as a fusion with the GAL4 DNA-binding domain, while another prey is cloned into pGADT7 and expressed as a fusion with the GAL4 activation domain. When bait and prey proteins interact, the GAL4 DNA-binding domain and the activation domain are brought together, activating transcription of 4 reporter genes (*HIS3*, *ADE2*, *lacZ* and *MEL1*) in a new yeast strain AH109. The *ADE2* reporter alone can provide a strong nutritional selection. When it is combined with *HIS3* selection, the incidence of false positives is greatly reduced. The following are the detailed protocols extracted from Clontech's Matchmaker GAL4 Two-Hybrid System 3 User Manual, Yeast Protocols Handbook and Yeastmaker Yeast Plasmid Isolation Kit User Manual. Materials prepared according to instructions of these manuals are underlined.

Subcloning genes into pGBKT7 and pGADT7

The genes of the major components of the *Pb01* cAMP signaling pathway have been cloned. They were then subcloned into bait vector pGBKT7 and prey vector pGADT7 for yeast two-hybrid analysis. ProofStart Taq polymerase was used for subcloning to secure the fidelity of gene sequences at the amino acid level. Sequencing was done to confirm that these genes were in frame with GAL4 DNA-

binding or activation domain. For yeast two-hybrid screening, random mutagenesis libraries were constructed using prey vector pGADT7 as described in section 2.14. Sequencing was done as well to make sure that these genes were in frame with the GAL4 activation domain.

Yeast transformation

A single colony of *S. cerecvisiae* strain AH109 was used to inoculate 50 ml of YPD or SD/-Trp (for sequential transformation). The culture was shaken at 250 rpm at 30°C overnight. A fraction of the overnight culture was transferred to 300 ml of YPD or SD-Trp to produce an OD_{600}=0.2. Cells were incubated at 30°C for several hours with shaking at 250 rpm until the OD_{600} reached 0.5. Cells were centrifuged at 1,000xg for 5 minutes at room temperature, and then washed with sterilized H_2O. Cells were precipitated again and re-suspended in 1.5 ml of freshly prepared 1X TE/LiAc to make competent cells. The following mixture was made:

Small scale simultaneous transformation	Large scale sequential transformation for library screening
0.1 μg of pGBKT7 construction	
0.1 μg of pGADT7 construction	20 μg of pGADT7 library
0.1 mg of Herring testes carrier DNA	2 mg of Herring testes carrier DNA
0.1 ml of competent cell	1 ml of competent cell
0.6 ml of PEG/LiAc	6 ml of PEG/LiAc

The mixture was incubated at 30°C for 30 minutes with shaking at 200 rpm. DMSO was then added to the mixture, using 70 μl for small scale transformation and 700 μl for large scale transformation. The cells were heat shocked at 42°C for 15 minutes. The cells were chilled on ice for 2 minutes then precipitated and re-suspended in TE (pH7.5). Cells were plated out on SD/-Leu/-Trp/-His/-Ade plates for selections, and

positive clones were observed 5 days after transformation. For library screening, positive transformants were restreaked on SD/-Leu/-Trp/-His/-Ade plates 2 times to allow loss of non-interacting prey constructions. Plasmids were isolated from the positive clones for further analysis.

Plasmid isolation from yeast strain AH109

Plasmids were isolated with Clontech's Yeastmaker Yeast Plasmid Isolation Kit according to the user manual. Positive clones were inoculated in 1 ml of SD-Leu/-Trp/-His/-Ade liquid medium for overnight growth. Cells were precipitated and broken with lyticase and SDS. Plasmids were purified on a CHROMA-SPIN-1000 column. This kind of plasmid, though not in high quality, was good enough for PCR amplification with a pair of gene specific primers to confirm that positives were truly brought by the gene tested. However, plasmids thus isolated contained both bait and prey constructions, and they were not good for sequencing. To segregate the plasmids, 5 μl of the plasmids isolated were used to transform NovaBlue *E. coli* competent cells, which were then subjected to either Amp^r selection for pGADT7 constructs or Kan^r selection for pGBKT7 constructs. Plasmids recovered from *E. coli* were highly pure and in larger amounts, and therefore suitable for DNA sequencing.

Chapter III

Results

3.1 Construction of Two cDNA Libraries of *Pb01*

Pb01 strain yeast cells were grown in modified-YPD medium at 37°C. Total RNA was isolated from the yeast cells using Qiagen's RNeasy Midi columns. About 1,700 μg total RNA was extracted from 3 grams of wet yeast cells. The quality of the total RNA was examined by eletrophoresis of 5 μg of total RNA sample on a 1% EtBr-agarose gel. Under the ultraviolet light, the total RNA lane was observed to cover 0-8 kb with obvious 18s and 28s rRNA bands. Poly(A)+ RNA was obtained from the 1,700 μg total RNA by using Qiagen's Oligotex mRNA Midi Kit. About 50 μg of mRNA was produced. The quality of mRNA was tested by running 4 μg of mRNA on a 1% EtBr-agarose gel. The mRNA lane was found to be a smear covering 0-8 kb, demonstrating its high quality without obvious degradation. The mRNA was then used to construct a λ-ZAP-cDNA library and a Creator pDNR-*Pb01*cDNA library using Stratagene's ZAP-cDNA library kit and Clontech's Creator SMART cDNA library construction kit, respectively.

The quality of the λ-ZAP-cDNA library and Creator pDNR-*Pb01*cDNA library was tested. Two parameters are important in examining the quality of cDNA library: the average size of the cDNA insertions and the titer of the original library before amplification. The λ-ZAP-cDNA library contained 40,000 independent phage plaques, indicating that its quality requires to be improved and another library with high original titer may be needed if a gene with low expression level is going to be cloned. The average size of the λ-ZAP-cDNA library was determined by PCR amplification of the cDNA insertions from individual plaques. As demonstrated in

Figure 3.1-1. Average size of cDNA insertions of *Pb01* λ-ZAP-cDNA library. Twenty randomly selected plaques were submitted to PCR with T3 and T7 primers. The marker on the right side lanes is the 1 Kb DNA Ladder from Invitrogen.

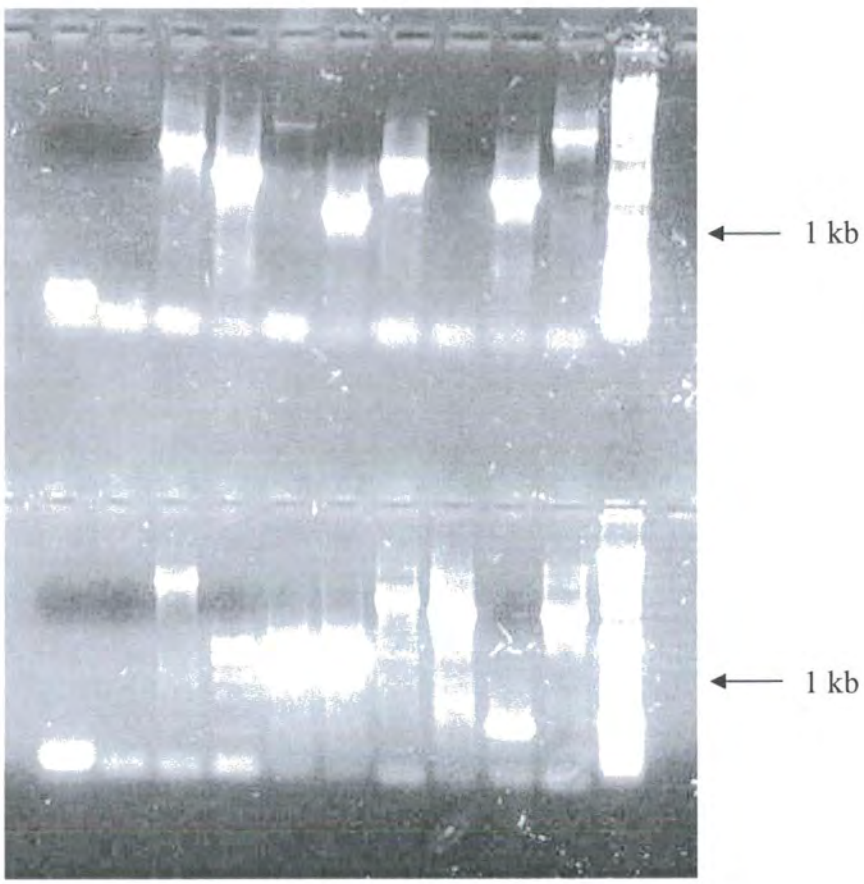


Figure 3.1-2. Average size of cDNA insertions of Creator pDNR-*Pb01*cDNA library. Thirty randomly selected independent clones were submitted to PCR with M13F and M13R primers. The marker in the middle of the gel is the 1 Kb DNA Ladder from Invitrogen.

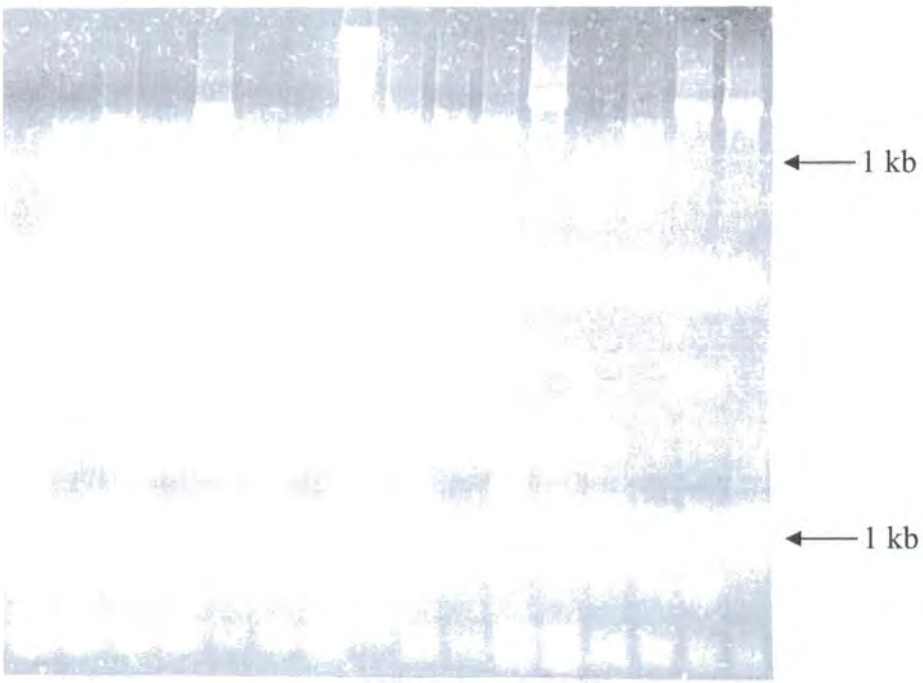


Figure 3.1-1, the average size of the cDNA insertions was more than 1 kb. The packaged λ-ZAP-cDNA library was amplified. The amplified λ-ZAP-cDNA library contained $3X10^8$ plaque forming units/ml. It was stored at -80°C.

The Creator pDNR-*Pb01*cDNA library contained 1,000,000 independent clones after transformation, indicating its high quality, which was confirmed later by cloning all the genes of *Pb01* in this thesis from it. The average size of cDNA inserts was demonstrated to be more than 1 kb after checking PCR products of 30 randomly selected independent colonies (Figure 3.1-2). All the transformants were pooled and the Creator pDNR-*Pb01*cDNA library plasmids were extracted with a final concentration of 1 μg/μl. The library plasmids were stored at -20°C.

3.2 Cloning of Adenylate Cyclase Gene *PbCYR1* from *Pb01*

The strategy for cloning the adenylate cyclase gene *PbCYR1* included library screening and PCR amplifications walking on both genomic DNA and cDNA. Primers were designed on the basis of immediate sequencing results. The cloning procedure is described as following and illustrated in Figure 3.2-1. Initially, two degenerate primers, PbAC-F1 and PbAC-R1, were designed that corresponded to conserved amino acid residues VFTDIKNST and MDY(YCF)GPMVN, respectively (Binz et al, 1998). A single 389-bp fragment, gPbAC-F1R1, was amplified from *Pb01* genomic DNA. This fragment was then labelled with fluorescein using Gene Images Random Prime Labelling Kit (Amersham) and used for screening the λ-ZAP-cDNA library. Approximately 6,000 plaques from the λ-ZAP-cDNA library were plated out and screened. After the first screening, 2 positive clones were obtained. They were purified to homogeneity after the third screening (Figure 3.2-2).

The inserts of the 2 clones were excised using ExAssist helper phage in SOLR cells (Stratagene), yielding pBSK plasmid containing insert. One of the 2 clones was named PbAC-2, having a 4.0 kb insert, and selected for sequencing. Sequence of PbAC-2 showed high homology to other fungal adenylate cyclases. However, it was a stretch of genomic DNA, indicating the existence of residual genomic DNA in the mRNA preparation before λ-ZAP-cDNA library construction. On the basis of PbAC-2 sequence, two specific primers, PbAC-F8 and PbAC-R30, were designed for downstream and upstream SSP-PCR. By using the 2 specific primers and 2 anchor primers (M13R1 and M13F1), 2 fragments, named cPbAC-R30M13F1 and cPbAC-F8M13R1-B6, were amplified from the Creator pDNR-*Pb01*cDNA library plasmids. These 2 fragments turned out to be cDNA as indicated by the existence of an intron-splicing and a poly(A) tail in the cPbAC-F8M13R1-B6 cDNA fragment. Another cDNA fragment, cPbAC-F15R19, connecting cPbAC-R30M13F1 and cPbAC-F8M13R1-B6, was amplified subsequently. An upstream cDNA fragment, cPbAC-R7SIV1, was amplified by SMART RACE PCR after RT reaction with PbAC-R7. Consecutively in the same way, two additional fragments from the 5' end of the cDNA were amplified, namely cPbAC-R12SIV1 and cPbAC-R21SIV2-12, and the start codon ATG was identified in the latter. Genomic DNA amplification was based on the sequence of the finished cDNA sequence. The genomic *PbCYR1* sequence was finished by amplifying a sequence, gPbAC-F12R30, from genomic DNA and another fragment gPbAC-R16AP21 from a GenomeWalker library by SSP-PCR.

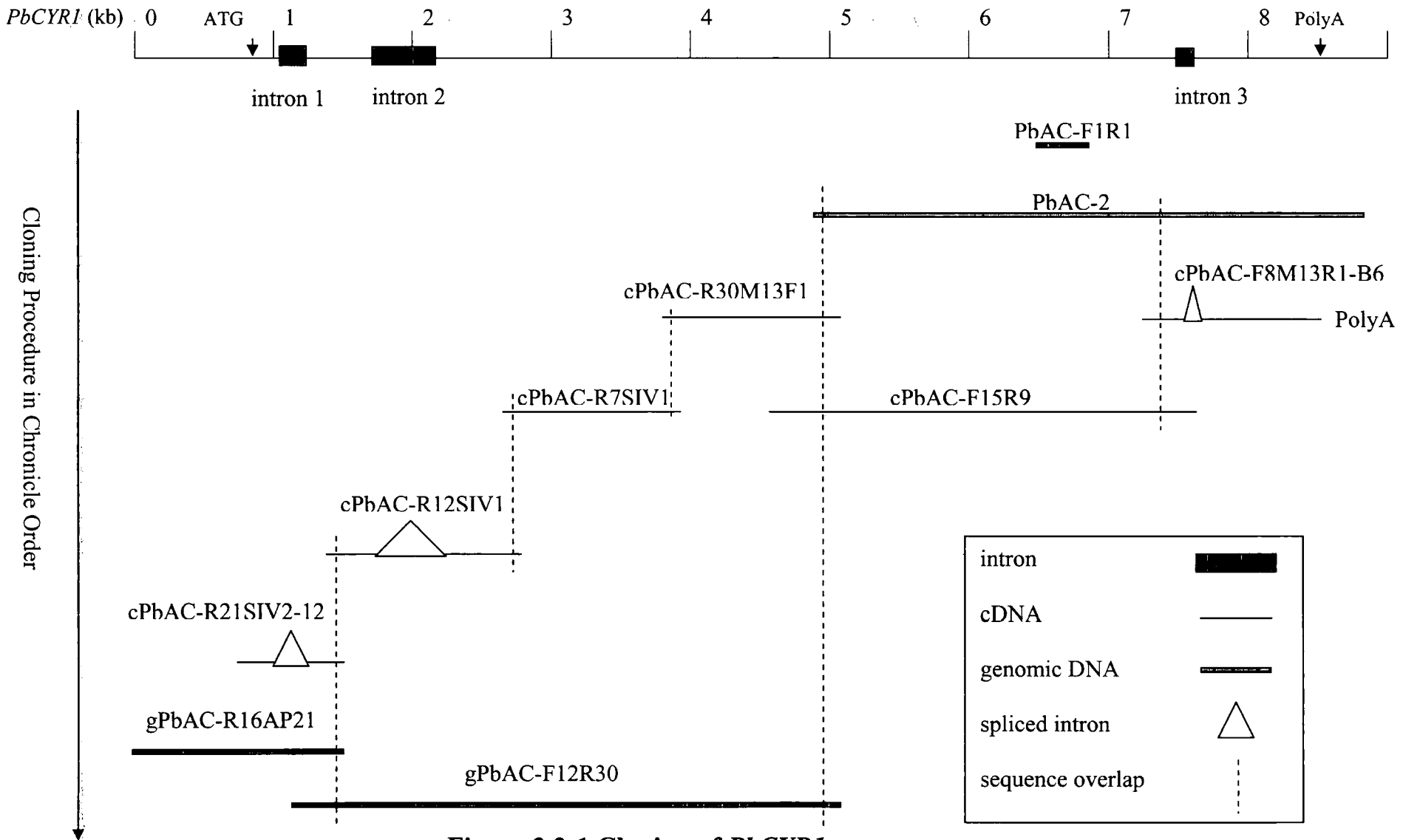
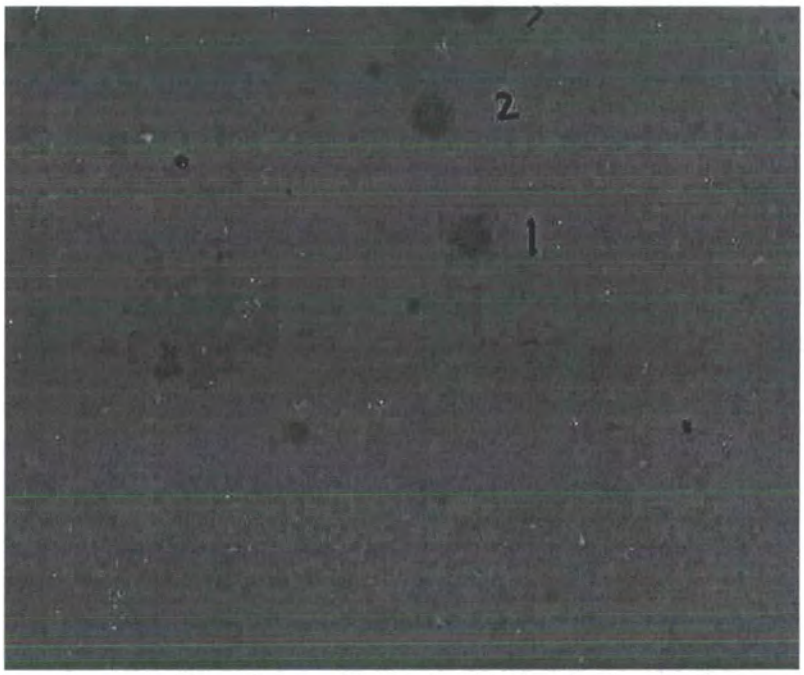
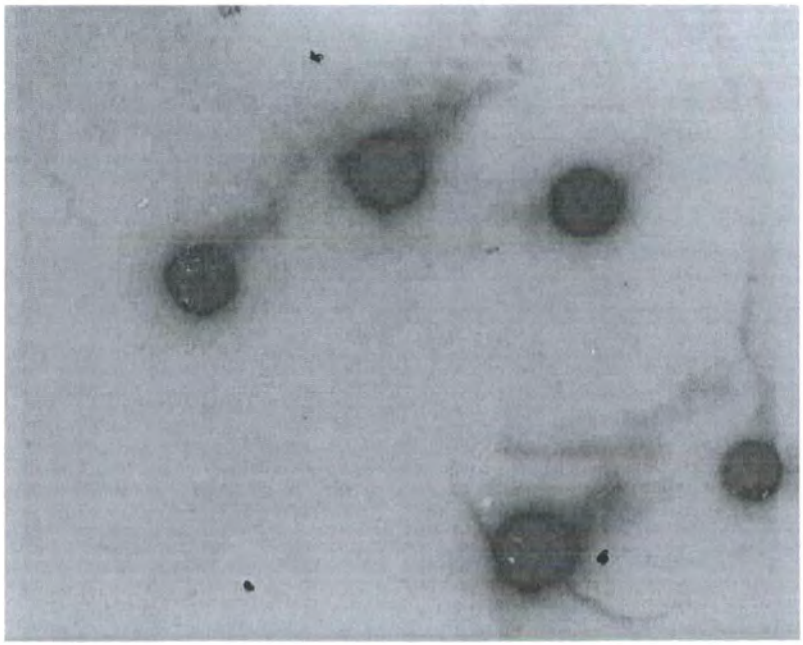


Figure 3.2-1 Cloning of *PbCYR1*

Figure 3.2-2. The first (upper panel) and the third screening (lower panel) of λ-ZAP-cDNA library with a fluorescin labelled gPbAC-F1R1 DNA fragment. Clone PbAC-2 was purified to homogeneity after the third screening.



Two positive clones obtained after the first screening



Positive plaques purified to homogeneity after the third screening

3.3 Nucleotide Sequence and the Deduced Amino Acid Sequence of Adenylate Cyclase *PbCYR1*

Following the successful amplification of fragments of cDNA and genomic DNA, the genomic DNA sequence, cDNA sequence and the deduced amino acid sequence of *PbCYR1* were determined and are illustrated in Figure 3.3-1. The *PbCYR1* gene can now be retrieved from GenBank with accession number of AY444997. This gene is very large, spanning more than 8 kb with 6,300 bp coding sequence interrupted by 3 introns. The first intron is 150 bp in length and located at nucleotide position 171-320; the second intron is 459 bp long and located at position 874-1,359; and the third is 74 bp long with position from 6,640 to 6,713 (Figure 3.3-1). The start codon ATG was determined by finding a Kozak consensus sequence (ANNATGG) at an ATG site and stop codons in the cDNA sequence upstream of it (Kozak, 1987; and Kozak, 1991). The gene encodes a protein of 2,100 amino acid residues with molecular weight of 234 kDa and predicted pI of 7.38.

In order to determine the initiation of transcription, RT-PCR was carried out on *Pb01* mRNA using a reverse primer PbAC-R22 (positions after the 2nd intron) in combination with different forward primers, which were designed on the basis of the 5'-noncoding genomic sequence, i.e. PbAC-F16, PbAC-F20, PbAC-F21, PbAC-F22 and PbAC-F23. Obvious bands were obtained when using primers PbAC-F16, PbAC-F20, PbAC-F21 and PbAC-F22. No amplification was observed when using primer pair F23 and R22, indicating that the initiation of transcription was located in the region between PbAC-F23 and PbAC-F22 (Figure 3.3-1 and 3.3-2). Potential promoter TATA boxes were found in that region. However, no CAAT boxes were found. A polyadenylation site was determined by observing a poly(A) tail in the

cDNA amplification (Figure 3.2-1 and Figure 3.3-1).

Fungal adenylate cyclase proteins are highly homologous. Figure 3.3-3 illustrates some important fungal adenylate cyclase proteins and their identity to PbCyr1. PbCyr1 is 53% identical to the adenylate cyclase AcyA of *A. fumigatus*, 50% identical to AnCyr1 of *A. nidulans*, 24% identical to Cyr1 of *S. cerevisiae* and 21% identical to SpCyr1 of *S. pombe*. A comparative alignment of adenylate cyclase catalytic domains of these fungi is illustrated in Figure 3.3-4.

PbCyr1, like other fungal adenylate cyclases, belongs to Class III adenylate cyclases. When a phylogenetic tree is created with catalytic domains of adenylate cyclases from different organisms, it is obvious that fungal adenylate cyclases and mammalian adenylate cyclases fall into 2 different groups and are very distantly related at the 2 ends of the tree (Figure 3.3-5). However, catalytic domains of fungal adenylate cyclases are relatively closely related to that of *Trypanosoma brucei*, the structure of which has been determined (Bieger and Essen, 2001).

The *P. brasiliensis* adenylate cyclase, when analyzed with SMART (EMBL), contains 4 domains, i.e. a Ras association domain (RA), 14 domains containing leucine-rich repeats (LRR_TYR domains), a serine/threonine phosphatase family 2C catalytic domain (PP2Cc domain) and an adenylyl/guanylyl cyclase catalytic domain (CYCc domain). A schematic illustration of the domains and their positions is given in Figure 3.3-6.

Figure 3.3-1. The nucleotide sequence and deduced amino acid sequence of *Pb* adenylate cyclase. Nucleotide positions are written in red numerals, amino acids in blue. Introns are denoted by lowercase letters with red colour. Potential promoter TATA boxes and polyadenylation site are underlined.

-1400 TATGTGTGTATGATTCTTTCGAACCCCCCTTTCTTGGTGATGAGCATGGC

-1350 AGTGCTACAGCTAGAGGGGGACCTGTAGCATTCGATCCCTACCCCCGATA

-1300 TACGGCCAAGTACACCTAGGCTTCGGCAGGAACATTGTCACCGCGAGTTA

-1250 AAGTCCGGAGTTGACGGTCGGCATCTCCTCTCTCGGGTTTGACATTAATA

primer PbAC-F23

-1200 AGTGGGGAATTGAGGTTGCGGTCACGCTACTGTTAGTTAGTCGAACTGGC

-1150 AGGGGAACTCTAGCTCATGGTCCCAGCGTTAACATCCCAGACAACTCCTG

-1100 GCGCGAATGCAACGGTTGGAGTCGGAGAGGTGTCTGTGGCTCTGTCTGTG

-1050 GCTCTGTCTGAGATTGTATACTTGATTGCTGGGTTGCGATTCAGGCTGAA

-1000 CATCTACTTGGCCCCCTCCTGTGATTCATATAGTGATCATATACTCCAGA

-950 ATTATACTCCAGAATTAGGGAGTTAAATTATGTTGACGTGAGAGAGAGTC

-900 CCGTGCGCTGACACAGCCTTGAGTAAGCGGACACGTGGGTCTAGCGCTTG

primer PbAC-F22

-850 CTTCGGGACAGGCCGAACCGGCCCCCATCGTTAGTCAGCAGCTGCCGGAA

-800 CGGCGTTGTTTCGCTGTTTGGCGGCTTGGACGTTGCTCTTTCGGGCTTGG

-750 ATGGGTCATTTCATTCTATCCCGATGTTATCATAAGTCAAACCATCATCA

-700 GTCTTCGAACATTATGCTTTAAACACCACCTCCCCATTCCCCAACATACC

-650 TGCCTGCTTCATGCTGCTTGGGGGTTTCTTCCGTCGTATGAACGGATGAA

-600 GCATAGCTCCTTATTTTAGCCTCTTTGTCCGCCTCACCCCCGTCCCCTCG

-550 CCTAGCTCTTACCCCGTTGTCCTTCCTTTGTCCCCGTATGTCCGGATATA

-500 TATGCACACACTGCAGGTTCTGAAATGGTTTAGCTTTGGGATACCGTCTG

-450 CTCTTCCTCTCGCCATCTCTGTCTCGTCGCTGTGTATCCCGTGGCCAACG

primer PbAC-F21

-400 GATTACGTTTGGAGCCAATGAGCCAGTTGGTTTCACCTTCGTAGCCTTTC

-350 CTAGATTTTCGTATCCACGTATCCCCAACCCGGGATTTGAGCTACTCACA

-300 GAGTTCATATTCGACCCCGAGCTTCGTGGCACTTCAAATCATATCTCCCC

-250 TACATCCCCCATCCAAGTCCCTTCCACGTCTCAATTCCACCCGCCACCCG

-200 CCTTGTCCAACCCAACATCTATCTACGCTGAATATCGGCCGGATGAATCA

-150 CATCCACTCGCGTCGTCTGAGAGTGTGAGAGGCGAAAGCTTCAACCCCCC

-100 GTCCAGCTCTCCATTCCCCCCCCATCCCTTTCCCCCCCCCCCCCTTCCAA

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-50   CGGGGCTTTTGATCTCCTTGGAGCGGCCAGAAACTTAGATTTGCGGCAGC
                                          primer PbAC-F17
  1   ATGGCAAGGAGACAGCGGGAGAAAGATAGGAACCTAAGGCGTGGTTCGGA
  1     M  A  R  R  Q  R  E  K  D  R  N  L  R  R  G  S
 51   AATGCCAAGTTCTCTCCTCTGGCGGTCCCCTGATGCGTTGCTGATATCGC
 17   E  M  P  S  S  L  L  W  R  S  P  D  A  L  L  I  S
101   AGGGTCTTGGCTTGGAAAGGGAACCCCGTCGCCCCAGTAGAGCTAGGGAG
 34    Q  G  L  G  L  E  R  E  P  R  R  P  S  R  A  R  E
151   ATTTCGGATCAGGATGACAGgtatgtttgatttcgtttttgaagaagcag
 51     I  S  D  Q  D  D
201   ttttgcttcgtgatgaattgttaggtcctgtttgggcacatcggaggcct

251   tcattctgttcaaaatgtggggaaacgctgttacgcaaacgttttatact

301   taatttttttttctttccagAAGGCTGAACTCGACATCCAGACACTCGTC
 57                        R  R  L  N  S  T  S  R  H  S
351   GTCCTCAAAGAGTAATCGTAACGCCCCCCGTAGGCCACATTCCCCCGATG
 66   S  S  S  K  S  N  R  N  A  P  R  R  P  H  S  P  D
401   TATCCCCAACAGACGTACCGACAACCAGCTTTGGAAACCATAAGAAAGCC
 84    V  S  P  T  D  V  P  T  T  S  F  G  N  H  K  K  A
451   CTTGCGATTCTTGGCGCATCGGATGACCCATCAGTCTCGCAAATGTTTCT
101     L  A  I  L  G  A  S  D  D  P  S  V  S  Q  M  F
501   ACAATACTCAAGCACAAATGCTCGGAAGCGGTCGCCATCATGGACACCAC
117   L  Q  Y  S  S  T  N  A  R  K  R  S  P  S  W  T  P
551   CCGCATCGTTAAAAACGACGGCTACTCGGGGAACCTTCTTTAACGATGGT
134    P  A  S  L  K  T  T  A  T  R  G  T  F  F  N  D  G
601   TCTGATGCGGAGGAAACCTCCCCTCAATTTCAGCTGGAGGCTGCCCTAAC
151     S  D  A  E  E  T  S  P  Q  F  Q  L  E  A  A  L
651   CGATCCCAGTAATTCCCCAGAACCCGTCCATGACGAACATCGCAGACCGT
167   T  D  P  S  N  S  P  E  P  V  H  D  E  H  R  R  P
701   CCGTTGCTAGCGCCACTACTGTTAGCAGTCAAGGATCGAAAGGCAGCGGT
184    S  V  A  S  A  T  T  V  S  S  Q  G  S  K  G  S  G
751   GGTCGTTTCCACAAACGCCTGCGCGGTCTCTTTGGGGATGAGCATGGCAG
201     G  R  F  H  K  R  L  R  G  L  F  G  D  E  H  G
801   CGCGTCAGATTCGGCTCTCCCGGAGCGGAGTCTGTCAAACCGAATTCCTA
217   S  A  S  D  S  A  L  P  E  R  S  L  S  N  R  I  P
851   TGTCATCTGGCAGTCAATCAAAGgtccgtgaaagaaataattcgacccca
234    M  S  S  G  S  Q  S  K
901   tctttgcacccccagagcgttcttcacccttggtcgtggcttccgacc

951   atgcactcctctaccttccagtgatgttacccttgaatgtatcaaaagc

1001  tcaatctaagatggcacgaaaagttccttcgttaatcggaatttacttca

1051  agggaagcctatatggtttcctgtcgttacatgttcccaactgcttcctt

1101  cggaaagtcttctgaaggtatctggataaaactgcacgacttgttgaaac

1151  tgcatattattcatcactagctctataaacccttttctcccttcattattt

1201  ggtttgcccatccctttctctaccattcctggctctctatttctaattag

1251  caccctcagggacattttcattatattctcgccatttcattctgaatta

1301  tactcgaactttaagctaatgtgggattgtagGATATATCAAACTATGGA
242                                     D  I  S  N  Y  G
1351  GATGCACCTGTGTATCCAGTTCCAGGAGACCCGGATGTTGAACGCTTTGG
248     D  A  P  V  Y  P  V  P  G  D  P  D  V  E  R  F
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1401  TTCCATGACAGATGGCGAGCGTGACTCAAAGAAAGATTCTCGTCTTCCTT
 264  G  S  M  T  D  G  E  R  D  S  K  K  D  S  R  L  P
1451  TCCACAGACATCGGCATGCTCGGAGTAAAGAAGAGAAGTTATCAGACCAA
 281   F  H  R  H  R  H  A  R  S  K  E  E  K  L  S  D  Q
1501  GGGTCTAACTTGCAACACATTTTCAGATCTTCAGACCATGACAACCACAA
 298    G  S  N  L  Q  H  I  F  R  S  S  D  H  D  N  H
1551  ACAAGTGTATAGTAAAGTAAAAGAGGGCACCCTCTCTCCAACTATCCCCA
 314  K  Q  V  Y  S  K  V  K  E  G  T  L  S  P  T  I  P
1601  CCGCTCTAGGAGCCCGCTCGATCAGTCCTTCGCCTAGTTTGCAGGGCGGC
 331   T  A  L  G  A  R  S  I  S  P  S  P  S  L  Q  G  G
1651  AGTGGGCAAGACTCTCAAGGGACAAGGTCGCCTGGAGGTTCATCCAACAA
 348    S  G  Q  D  S  Q  G  T  R  S  P  G  G  S  S  N
1701  GCGATCATTTTTTACGAAGATTCGACGCCATCCAAAACCTCCAATCGGGA
 364  K  R  S  F  F  T  K  I  R  R  H  P  K  P  P  I  G
1751  GTAGTCTTCGGGGTATGCCATCTTCGAAATCTGCCTACGACGGTGGTGGG
 381   S  S  L  R  G  M  P  S  S  K  S  A  Y  D  G  G  G
1801  AAGGGATCTAGGTTTTTGAAACGAGATATGTCTCCTGCTAGATATGGTCG
 398    K  G  S  R  F  L  K  R  D  M  S  P  A  R  Y  G
1851  GAATTCGAGCTTTGATAGTGTCACCAATACAAAGCCGTGGGAAAGTGTCG
 414  R  N  S  S  F  D  S  V  T  N  T  K  P  W  E  S  V
1901  AGTCTGACCGCAAAAAGGAGTCTGCGAAGGGGGTATCCAAGTTCCGCCAT
 431   E  S  D  R  K  K  E  S  A  K  G  V  S  K  F  R  H
1951  CCGCGTTTTCCGTTTGGTCATGATACTGGAAATAAGGATGACAAAACACA
 448    P  R  F  P  F  G  H  D  T  G  N  K  D  D  K  T
2001  CCAAGATAATTTTGAACAAGATCGGATCTGGAAGTTAGATACCGATCTGA
 464  H  Q  D  N  F  E  Q  D  R  I  W  K  L  D  T  D  L
2051  CAAATCTCTCTGGTATCGTCGCACAACCTCAACCTTTATCCCCTACCGAT
 481   T  N  L  S  G  I  V  A  Q  P  Q  P  L  S  P  T  D
2101  TGTGGTGGTATATATACTGGAACTCCACTTCCTGAGCATAAGGAAAGAGA
 498    C  G  G  I  Y  T  G  T  P  L  P  E  H  K  E  R
2151  ATTCCGCAATCCCTTTGAACCCTCCAGCACTGGTGACTGGCATGCTCCAG
 514  E  F  R  N  P  F  E  P  S  S  T  G  D  W  H  A  P
2201  AGAGCTGGGCTGTGAGGAAAGTTGGGGATGACATTTTGTCCCGGCTTCCT
 531   E  S  W  A  V  R  K  V  G  D  D  I  L  S  R  L  P
2251  GAGCTCACTGATGATGGCGGGGCGATGGTAGACGACGAAGGGCGACCGTA
 548    E  L  T  D  D  G  G  A  M  V  D  D  E  G  R  P
2301  CTGTGTCCGCATCTTTCGCATCGACTCTACATTTACAACTCTTTCCTCTA
 564  Y  C  V  R  I  F  R  I  D  S  T  F  T  T  L  S  S
2351  ATCTGAACACTACCGTGACCGAAATACTGGAAATGCTTGGCCGCAAGTCG
 581   N  L  N  T  T  V  T  E  I  L  E  M  L  G  R  K  S
2401  TTCTTGCAAGATGAGCTGAATAACTACGATATAGTCGTACGGAAGAATGA
 598    F  L  Q  D  E  L  N  N  Y  D  I  V  V  R  K  N
2451  CCTTTCAAGAACTCTGGACTACGGGGAGAGGCCGATTCTCATGCAAAAGA
 614  D  L  S  R  T  L  D  Y  G  E  R  P  I  L  M  Q  K
2501  AATTGCTCGAGCAGGCTGGATATCAACCATCAGACCGTATAGCGGATATT
 631   K  L  L  E  Q  A  G  Y  Q  P  S  D  R  I  A  D  I
2551  GGTCGAGAGGATAATAGCTACCTCTGCTGTTTCACATTCCTTCCCACGAA
 648    G  R  E  D  N  S  Y  L  C  C  F  T  F  L  P  T
2601  ACTTTCGGGCTATTCGAGTCTGGATGCTGACTTGGGAATCAACGAGAACC
 664  K  L  S  G  Y  S  S  L  D  A  D  L  G  I  N  E  N
2651  AAAAGTTTAGCCACGTCGACTTGCACGGGCGCAGTCTCATCACCCTGCCC
 681   Q  K  F  S  H  V  D  L  H  G  R  S  L  I  T  L  P
2701  ATCACACTCTATAAAAGGGCGAGTGAGATCATATCCTTGAATCTTTCGAG
 698    I  T  L  Y  K  R  A  S  E  I  I  S  L  N  L  S
2751  AAATTTAGCCTTGGATATTCCGAAGGATTTTATTCAAAGCTGCATCAACC
 714  R  N  L  A  L  D  I  P  K  D  F  I  Q  S  C  I  N
2801  TCCGTGAATTAAAATATATCGGAAACGAGGCATGGCGTCTTCCGGCAAGC
 731   L  R  E  L  K  Y  I  G  N  E  A  W  R  L  P  A  S
2851  TTAAGCTTGGCGACGCGGCTTACATATCTAGACATATCCAACAATAGGCT
 748    L  S  L  A  T  R  L  T  Y  L  D  I  S  N  N  R
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2901  TGAACAGCTGGATAATGCAGAATTACACAATTTGCATGGACTTGTCAGCT
 764  L  E  Q  L  D  N  A  E  L  H  N  L  H  G  L  V  S
2951  TGAAAATGTCGAATAATAACCTCTCGACGCTGCCTGCTTATTTTGGAGAC
 781   L  K  M  S  N  N  N  L  S  T  L  P  A  Y  F  G  D
3001  TTCCCTGCATTACGAAGCCTGAATATTTCTTCCAATAGTTACCGCACTTT
 798     F  P  A  L  R  S  L  N  I  S  S  N  S  Y  R  T
3051  TCCCGAGTGTTTATACAATCTGAAGAGTCTCGTTGATCTTGATATCAGCT
 814  F  P  E  C  L  Y  N  L  K  S  L  V  D  L  D  I  S
3101  TCAACAAAATCTCCGAGTTGTCGGATATTGGACGCCTTACGACACTGGAA
 831   F  N  K  I  S  E  L  S  D  I  G  R  L  T  T  L  E
3151  CGGTTATGGGTGACTAACAATGGCTTGCATGGCCCGCTGGGTGAGACTTT
 848     R  L  W  V  T  N  N  G  L  H  G  P  L  G  E  T
3201  TAGAGATCTTGTCAATCTCAAAGAGATAGACGCACGATTCAACAGCATCA
 864  F  R  D  L  V  N  L  K  E  I  D  A  R  F  N  S  I
3251  CTAGTATAGATAACATCACTCACCTTCCACGACTAGAGCGGTTGTTGATT
 881   T  S  I  D  N  I  T  H  L  P  R  L  E  R  L  L  I
3301  GGCCATAACAGCGTGTCCACATTTTCAGGCTCTTTCATGAAGCTGCGCAC
 898     G  H  N  S  V  S  T  F  S  G  S  F  M  K  L  R
3351  TCTTGCCCTGGACCATTGCCCAGTCACCGAGTTTGATCTCACTAGCCCCC
 914  T  L  A  L  D  H  C  P  V  T  E  F  D  L  T  S  P
3401  TTCCGACTCTCACCTCTCTCAACATCGCGTCAGCCAAACTTGTTCAATTC
 931   L  P  T  L  T  S  L  N  I  A  S  A  K  L  V  Q  F
3451  AAGGATACCCTTTTTACCAATATCCCACACCTGACCAAACTTATTCTTAA
 948     K  D  T  L  F  T  N  I  P  H  L  T  K  L  I  L
3501  TAAGAATCATTTCGTTACTCTCTCCTCCTATATCGGAACCCTCCGAAAAC
 964  N  K  N  H  F  V  T  L  S  S  Y  I  G  T  L  R  K
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3601  ATAGGATGTCTAACAGAATTGAAGTGCCTCAACCTACGCGAATGTAATCT
 998     I  G  C  L  T  E  L  K  C  L  N  L  R  E  C  N
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1014  L  N  K  L  P  S  E  L  W  Y  C  S  K  L  E  T  L
3701  ATGTGTCGTCTAATGTCCTTGACGCCTTTCCAAAGATCGCTGGCTCACCT
1031   N  V  S  S  N  V  L  D  A  F  P  K  I  A  G  S  P
3751  CCTATTCCTCCAAACGAATATCATGGAAATGGTACTCCCGCAACCACTCC
1048     P  I  P  P  N  E  Y  H  G  N  G  T  P  A  T  T
3801  TGGTCTGGCTTCGCCAAGTCACGAAGAACTTGGAAAGCTGGAGGATTTCG
1064  P  G  L  A  S  P  S  H  E  E  L  G  K  L  E  D  F
3851  AAGCCAGACGACCAAGTCATTCCTCCAGTGGTCTTCTGAGCGTGGCAAGT
1081   E  A  R  R  P  S  H  S  S  S  G  L  L  S  V  A  S
3901  TCCTCCGCTAATTCATCAAATAGAAAAGGCTCAATTGTCTCGATGCCTCA
1098     S  S  A  N  S  S  N  R  K  G  S  I  V  S  M  P
3951  TAACCCGACTGTAAAGAAGTCTTCTGTCATATCTCGAGCTGCAACTGAAA
1114  H  N  P  T  V  K  K  S  S  V  I  S  R  A  A  T  E
4001  CCCCATCCTCGCGCAAGGATTCAACCTTCTCGCAAAGAATATCGTTTACG
1131   T  P  S  S  R  K  D  S  T  F  S  Q  R  I  S  F  T
4051  TTTGCGGGATCTCTGAAGCAACTTTATCTTGCAGACAATCGTCTTGAAGA
1148     F  A  G  S  L  K  Q  L  Y  L  A  D  N  R  L  E
4101  CGATGTATTTCAACAGTTGGCCTTTTTGGTGGAAATCCGCATCTTGAACT
1164  D  D  V  F  Q  Q  L  A  F  L  V  E  I  R  I  L  N
4151  TGTCCTATAACGAGTTGACGGAGATTCCGCAGGGTCTCCTTAGACGGTGG
1181   L  S  Y  N  E  L  T  E  I  P  Q  G  L  L  R  R  W
4201  CAGTATTTGGTGGAGCTGCATGTCTCAGGAAATCAACTGAGCGCGCTTCC
1198     Q  Y  L  V  E  L  H  V  S  G  N  Q  L  S  A  L
4251  CTCGGACGACCTTGAAGAATCAAGTAGTCTAAAGGTATTGCACATAAACG
1241  P  S  D  D  L  E  E  S  S  S  L  K  V  L  H  I  N
4301  GCAATAAATTCCAGGTACTTCCTGCGGAGCTCTGTAAGGTGAATCGCCTT
1231   G  N  K  F  Q  V  L  P  A  E  L  C  K  V  N  R  L
4351  GCGATTTTAGATGTGGGGAGTAATTCTCTAAAATACAATGTTTCCAACTG
1248     A  I  L  D  V  G  S  N  S  L  K  Y  N  V  S  N
```

```
4401  GCCGTACGATTGGAATTGGAATTGGAATCGCAATTTAAAATACCTGAATT
1264  W  P  Y  D  W  N  W  N  W  N  R  N  L  K  Y  L  N
4451  TCTCGGGCAACAAGCGATTTGAGATCAAACCCAATACCTCTTATACTTCT
1281   F  S  G  N  K  R  F  E  I  K  P  N  T  S  Y  T  S
4501  GGGATTCCTGCCGTTAATAGCACAGATCTGACCAACTTCAACTCTTTGAA
1298     G  I  P  A  V  N  S  T  D  L  T  N  F  N  S  L
4551  ACATCTTAGAGTCCTGGGGTTGATGGATGTTACTCTTACTATCCCTACCA
1314  K  H  L  R  V  L  G  L  M  D  V  T  L  T  I  P  T
4601  TCCCTGACCAGACTGAGGATAGGAGAGTGCGGACGTCATCATCCTTGTCT
1331   I  P  D  Q  T  E  D  R  R  V  R  T  S  S  S  L  S
4651  GGCTCTCTTGCGTATGGTATGGCAGATAGCCTTGGGCGAAACGAACATCT
1348     G  S  L  A  Y  G  M  A  D  S  L  G  R  N  E  H
4701  ATCAATAGTTGATATGCTTGTTACTCGGTTCCGAGGCAACCAGACGGAGA
1364  L  S  I  V  D  M  L  V  T  R  F  R  G  N  Q  T  E
4751  CGGTTCTCGGTTTATTTGACGGCCAGTCCTCTTCTAGCAGCGGATCAAGG
1381   T  V  L  G  L  F  D  G  Q  S  S  S  S  S  G  S  R
4801  ATAGCGAAATACCTTCAAGAGCACTTCACTACATCGTTTACAGAAGAGTT
1398     I  A  K  Y  L  Q  E  H  F  T  T  S  F  T  E  E
4851  GAAAAAGCTTCGTCCATCAGGAGAAACCCCGCTCGATGCACTGCGGAGGA
1414  L  K  K  L  R  P  S  G  E  T  P  L  D  A  L  R  R
4901  CCTTTTTGGGACTCAACAAGGATATAGCTACGGCTGCTCAACGGTCTTCC
1431   T  F  L  G  L  N  K  D  I  A  T  A  A  Q  R  S  S
4951  ATGGATGATCGTGAGCTCCATCATTTTGAACGTGGGACAACAACATCTAG
1448     M  D  D  R  E  L  H  H  F  E  R  G  T  T  T  S
5001  CAAGCTGCTCACGAAGGATGACTATAATCTAGGAGGCGTAGCGACTGTCC
1464  S  K  L  L  T  K  D  D  Y  N  L  G  G  V  A  T  V
5051  TGTATCTGCAAAATATGGAGTTGTTTGTTGCAAATGTTGGGGATGCTCAA
1481   L  Y  L  Q  N  M  E  L  F  V  A  N  V  G  D  A  Q
5101  GCGGTTCTTGTGCATTCGAATGGGGATTTCAAGTTTTTGACTTGTAACCA
1498     A  V  L  V  H  S  N  G  D  F  K  F  L  T  C  N
5151  CGATCCTGCTGAGGCTTCGGAACGGGAACGTATCCGGGCAGCTGGCGGGT
1514  H  D  P  A  E  A  S  E  R  E  R  I  R  A  A  G  G
5201  TTGTTTCCCGCAATGGAAAATTGAATGATAAGCTATCTGTGTCTCGGGCC
1531   F  V  S  R  N  G  K  L  N  D  K  L  S  V  S  R  A
5251  TTTGGATATTACACAATGATGCCTGCGGTTATTGCGGCTCCTAGCACCCG
1548     F  G  Y  Y  T  M  M  P  A  V  I  A  A  P  S  T
5301  GAAAGTTACTTTGACCGCCCAGGACGAAATGATAATTCTTGCGTCAGGCG
1564  R  K  V  T  L  T  A  Q  D  E  M  I  I  L  A  S  G
5351  AGTTGTGGGATTATGTTACACCGGACGTTGTTGTTGATGTTGCAAGATCT
1581   E  L  W  D  Y  V  T  P  D  V  V  V  D  V  A  R  S
5401  GAACACGGAGACTTGATGATTGCCTCTCAGAAGCTTCGTGACTTGGCCAT
1598     E  H  G  D  L  M  I  A  S  Q  K  L  R  D  L  A
5451  TGCCTATGGTGCTACCAACAAAATCATGGTGATGATTGCTGGTGTCAGTG
1641  I  A  Y  G  A  T  N  K  I  M  V  M  I  A  G  V  S
5501  ATCTTAAACGACGGGGTGAACGTCCCAAGCTCAGAAGTCACAGTATCTCC
1631   D  L  K  R  R  G  E  R  P  K  L  R  S  H  S  I  S
5551  ATGGGACTGTCTCCATTAACTGACGAACATCTCTTCACCACACCAAGTAA
1648     M  G  L  S  P  L  T  D  E  H  L  F  T  T  P  S
5601  GATCAAGAAACGTGGCAGACCAGGCGACTCCAGGTTAGCACGTTTGGATC
1664  K  I  K  K  R  G  R  P  G  D  S  R  L  A  R  L  D
5651  GCGTGGAAGCCCCTACTGGCGAATTGGCGATCATTTTCACTGATATTAAG
1681   R  V  E  A  P  T  G  E  L  A  I  I  F  T  D  I  K
5701  AAATCCACGTCGCTTTGGGAGACGTATCCCGTTGCCATGCGCTCTGCAAT
1698     K  S  T  S  L  W  E  T  Y  P  V  A  M  R  S  A
5751  TCAAATTCATAATGATCTCTTCCGTCGACAGTTGGCTCAGATAGGTGGAT
1714  I  Q  I  H  N  D  L  F  R  R  Q  L  A  Q  I  G  G
5801  TTGAAGTGAAAACAGAAGGTGACGCTTTCATGGTCTCCTTCTCCACGGCC
1731   F  E  V  K  T  E  G  D  A  F  M  V  S  F  S  T  A
5851  ACCGCAGCGTTACTCTGGTGCTTCACCTGCCAAAGCCAACTTCTTGAAGC
1748     T  A  A  L  L  W  C  F  T  C  Q  S  Q  L  L  E
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5901   GGAATGGCCGACGGAGATCATGGAATCTCCTGCTTGCAGGCCACAGTATG
1764   A  E  W  P  T  E  I  M  E  S  P  A  C  R  P  Q  Y
5951   ACTCGGATGACAACATCATATACCGCGGGCTGTCTGTGCGAATGGGAGTA
1781    D  S  D  D  N  I  I  Y  R  G  L  S  V  R  M  G  V
6001   CACTGGGGAACGCCTGTCTGTGAACAAGATCCTGTTACTGGTCGAATGGA
1798      H  W  G  T  P  V  C  E  Q  D  P  V  T  G  R  M
6051   TTACTTTGGCCCCATGGTAAACCGTGCCTCCCGCATTTCCGCAGTTGCGG
1814   D  Y  F  G  P  M  V  N  R  A  S  R  I  S  A  V  A
6101   ATGGTGGGCAAATCTTCGTTTCAGCCGACTTCGTTGCTGAAATTCAACGG
1831    D  G  G  Q  I  F  V  S  A  D  F  V  A  E  I  Q  R
6151   AATCTTGAAACATTCGCGGATTATGATCGGATCGGCTCTATGGATTCTTC
1848      N  L  E  T  F  A  D  Y  D  R  I  G  S  M  D  S
6201   TGAGGATAATTACATTGACGACTATCTTAATCACAATATCCGTCGCGAGC
1864   S  E  D  N  Y  I  D  D  Y  L  N  H  N  I  R  R  E
6251   TTTATCAACTAAGCACCCAGGGCTTTGAGGTAAAGGATATGGGTGAGCGG
1881    L  Y  Q  L  S  T  Q  G  F  E  V  K  D  M  G  E  R
6301   AAACTGAAGGGGCTTGAGAATCCTGAATTCGTTTACTTGATGTATCCACA
1898      K  L  K  G  L  E  N  P  E  F  V  Y  L  M  Y  P
6351   CTCTCTTGCTGGACGCCTCAACGCTCCACGGGCTGAGATTGAAGAGGCAG
1914   H  S  L  A  G  R  L  N  A  P  R  A  E  I  E  E  A
6401   ACACACTTCCCATTCCCTTGGGTCAAGACAAGCATCTCAGTATTGAAGCG
1931    D  T  L  P  I  P  L  G  Q  D  K  H  L  S  I  E  A
6451   GATCTAGTCTGGCGCCTGTGGACTGTTGCTTTACGTTTGGAGGCATTTTG
1948      D  L  V  W  R  L  W  T  V  A  L  R  L  E  A  F
6501   CAGTGCCCTGGAAAATTTCGGAGAGATTGGTCTTCGGCAGCCGGAGATGG
1964   C  S  A  L  E  N  F  G  E  I  G  L  R  Q  P  E  M
6551   GCCTGATTGATGCAGTCAAGCAGCGCCCTGGGGAACTTTCGGATGCGGTA
1981    G  L  I  D  A  V  K  Q  R  P  G  E  L  S  D  A  V
6601   GTGTTGAGTCTAATTGAACATCAGGTTACGCGGGTGGAGgtgagttactc
1998      V  L  S  L  I  E  H  Q  V  T  R  V  E
6651   ttccccacttacactactattctatattgtagactaaactaacgcaacgg

6701   aaacccgttatagACATGTGCAAATACCCTCACAATCCGGCACATGATGA
2011                  T  C  A  N  T  L  T  I  R  H  M  M
6751   GGCCTTTCAAACGCGGAAACTCTCTCAAAGATATCGCCGTTCCCATGTCG
2023    R  P  F  K  R  G  N  S  L  K  D  I  A  V  P  M  S
6801   GATATTCTCAATCAGTTGAAGGTGCAGTTGAGCGAATTTAGAGCTCTAAA
2040      D  I  L  N  Q  L  K  V  Q  L  S  E  F  R  A  L
6851   GGAGTCAATAAGTCCCATCGCCGACTCTCAGGATCCCCAACAAAGCCTTG
2056   K  E  S  I  S  P  I  A  D  S  Q  D  P  Q  Q  S  L
6901   CGGTGTTGAAGAGCCCCATACAGAGCTCGTCAGCCTTATCTGATATTATG
2073    A  V  L  K  S  P  I  Q  S  S  S  A  L  S  D  I  M
6951   GCCGTTGGTGGTTCTAGTTCTTCGAGCACGGCGTAAAGCTTTATAAAACC
2090      A  V  G  G  S  S  S  S  S  T  A  *
7001   TGCCACAGGAACAGCTGCTTTATGAATGGAATCGAAGTCCTTGACATCAA

7051   TTCTTTTATGCTCTTACTCAACTAGCGCCGATCGGATTGCAAGTCGTCCG

7101   GAGTTATTCTCTGTTCCACCCTTTTTTTTTTCGCTTCCCCTTTTTCTCTT

7151   TCGTCCGCTTTTGTTCTCTTTATCCCGGCCGTTGCCTATGACTTGATGTC

7201   CGTTTGATTTAATTCGTGGGGAAAAGCAGCGTATTTGGAATTTAATTACC

7251   ACCTTGCCGTATTACTTTCTGCCATATTTTGGTTTATTTTTTTCATTTAC

7301   TTTATTTACTTAATTTTGGGTGTGGGGGGTTTGCGTCTGTTTGCTTGGTA
                                       primer PbAC-R32
7351   CGATCGATATTGTTTCCATAAAGTGCTTCTTTCTCTCTGGCCGGTTCCAT

7401   CGTTCATTTCTGGGATTACACCATTTTATTTTTGCTAAGATCGGCGTATG
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7451 TGGCTGCTGGAAATGAGGAGTATATTATCTTATCCCCATTTATCTATCAT

7501 TATAATTTGGTGAGCCTTTGGTATATTTACAGGTAATTTTGGTGCCAATG

7551 AAGGAATTTTTTTGCTCTCTTCGTTTTCGTGCAAAAAGGGATCCGTGGCT

poly(A) adding position

7601 GGTGCAGGATGACGTAAAGTCACATGACATACCTGACCTGTGGAGTTAAT

7651 ATGTGGCGCGGCGCCGCGCATCGCATCTTGTTTTTTGTTGCTTTCTTGTT

7701 GTCGCCGCCATCCAAATTGCATCAAAGCTACATCTCTCCACTCCATCATT

7751 CCATCCAGGGTCTTTTCAACTTGGCCTCCGGATTCGTCTTCTTAAAATAC

7801 ACTCTCTTATCTGCAGCTTTTGGTGCACGTGAATTTGTATTGATGCCGTA

7851 GTTGCAGCGTGGATTTTTCCTGCTGTTGCCCTGGATACGGAACGCTTCAC

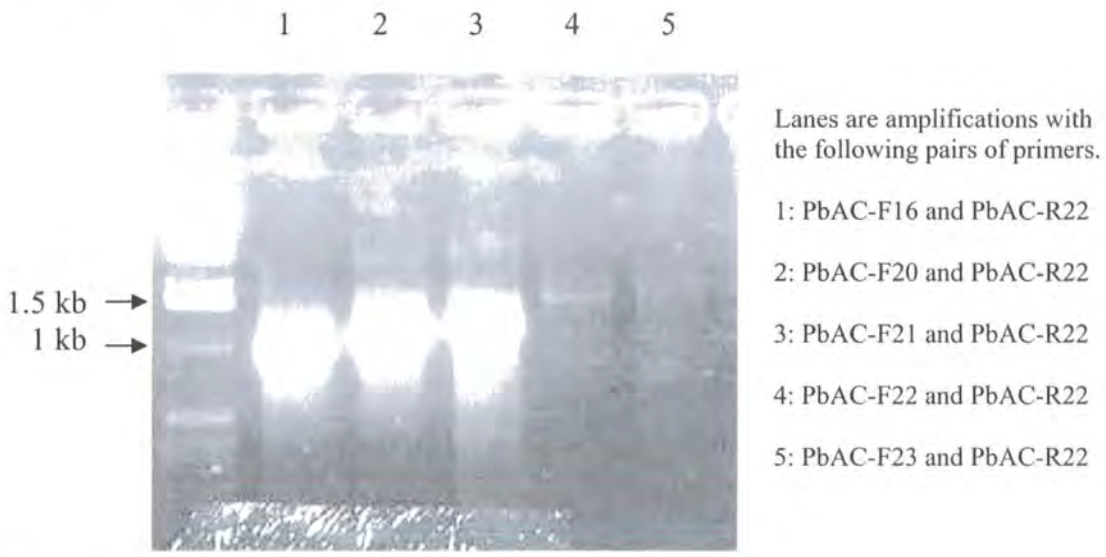
7901 ACGCACTTAATTTATTGGCTCAACCTTCTTGAGTAAAGCGAAACAATTTT

7951 TTTTTTTTTTTCCTGAGAGATACACCG

Figure 3.3-2. Determination of transcription initiation site of *PbCYR1*.
A: RT-PCR products after amplification with different pairs of primers. Bands can be observed when using primer pairs "PbAC-F16 and PbAC-R22", "PbAC-F20 and PbAC-R22", "PbAC-F21 and PbAC-R22", and "PbAC-F22 and PbAC-R22". No band was amplified when using "PbAC-F23 and PbAC-R22".

B: A schematic diagram illustrating the PCR reactions in A. The predicted transcriptional initiation site was located at -1238 to -908 (referred to figure 3.3-1).

A: RT-PCR products on agarose gel



B: Schematic illustration of A

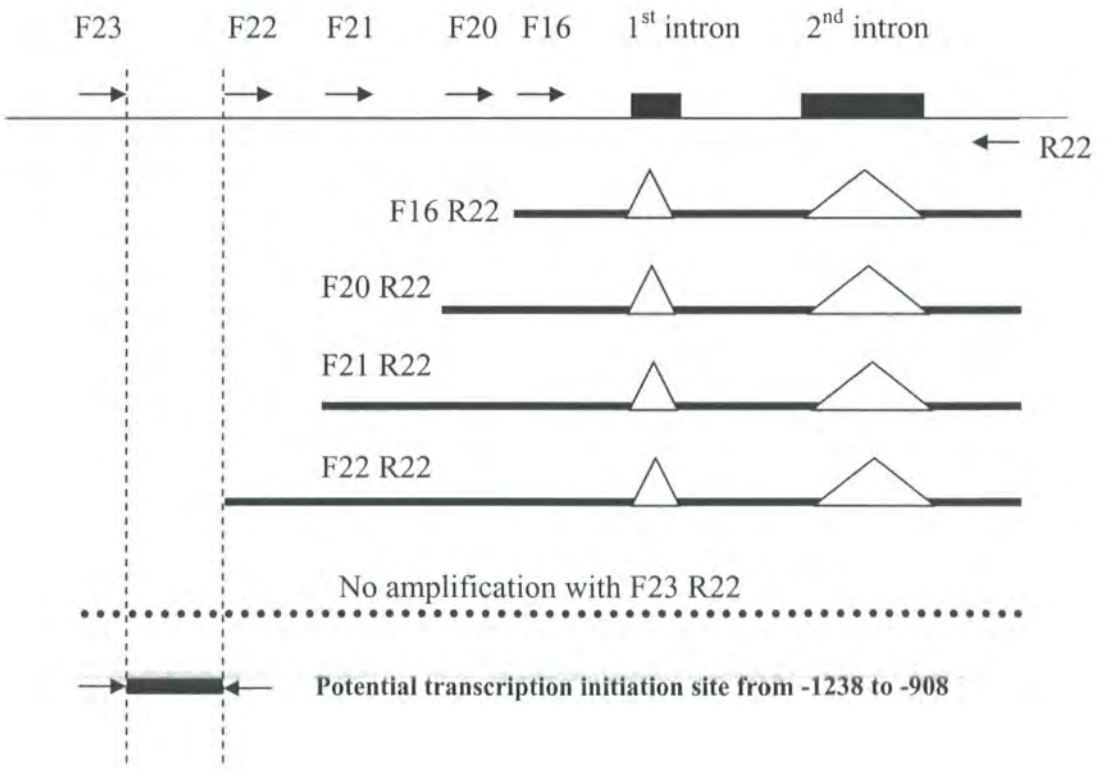


Figure 3.3-3. Homology of fungal adenylate cyclase proteins.

GenBank Accession Number	Protein: Organism	% identity to PbCyr1
AAS01025	PbCyr1: *P. brasilliensis*	
CAC81748	AcyA: *A. fumigatus*	53%
EAA59222	AnCyr1: *A. nidulans*	50%
AAC34139	Mac1: *M. grisea*	39%
XP_329423	NcCyr1: *N. crassa*	34%
BAA93553	CaCyr1: *C. albicans*	27%
P49606	Uac1: *U. maydis*	24%
CAA89295	Cyr1: *S. cerevisiae*	24%
EAL19815	CnCyr1: *C. neoformans*	24%
CAA19571	SpCyr1: *S. pombe*	21%

Figure 3.3-4. Comparative alignment of adenylate cyclase catalytic domains. A multiple amino acid sequence alignment was performed using VectorNTI 6.0 (Informax). GenBank accession numbers of proteins are given following the names of the proteins. The abbreviations are: Pb, *P. brasilliensis*; Af, *A. fumigatus*; An, *A. nidulans*; Sc, *S. cerevisiae*; Sp, *S. pombe*.

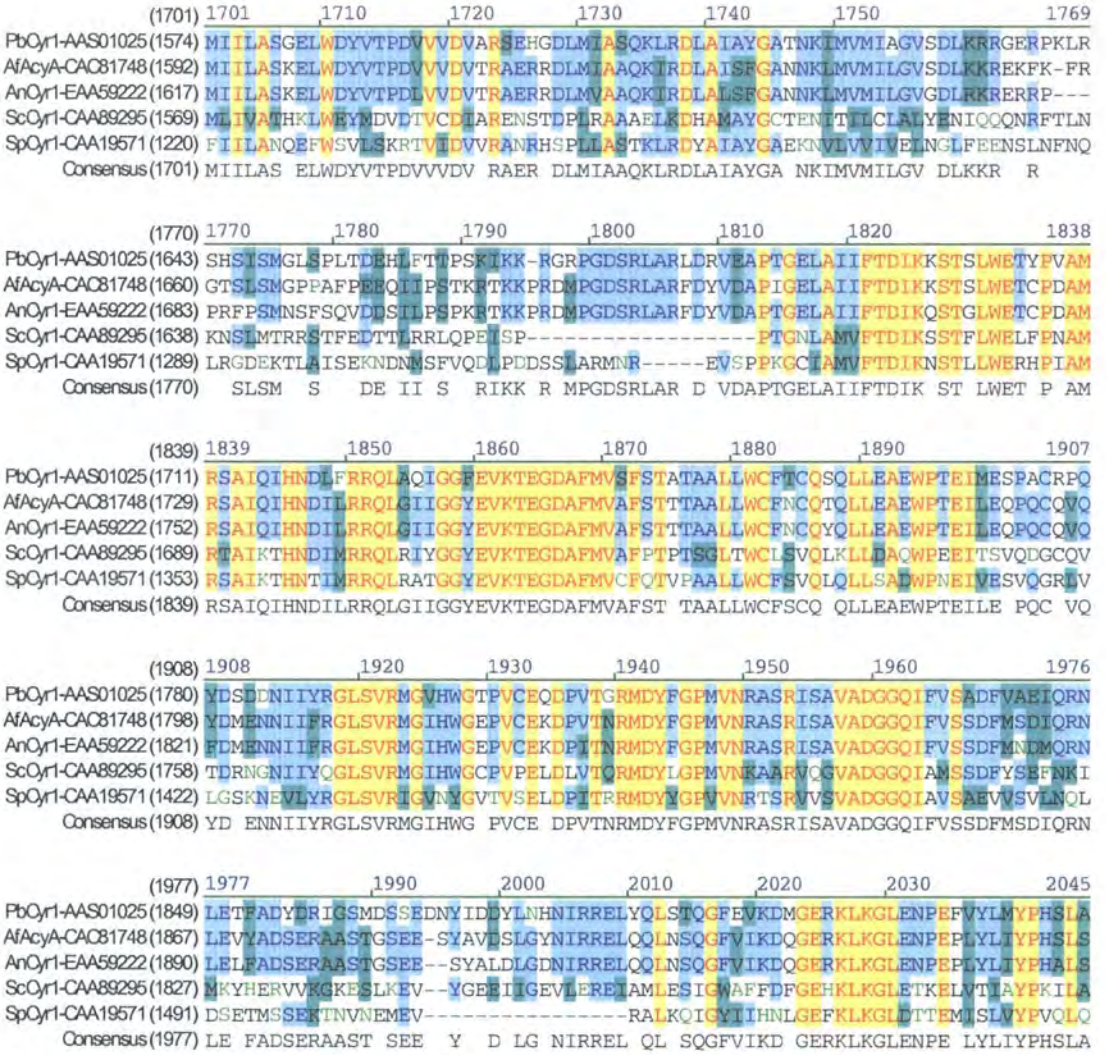


Figure 3.3-5. Phylogenetic relationship of Class III adenylate cyclases. The tree was constructed by using Vector NTI 6.0 (Informax). Only catalytic domains were used in the phylogenetic analysis (for mammalian adenylate cyclases, it is the 2nd catalytic domain that was used). The protein GenBank accession numbers and the amino acid positions are bracketed. The abbreviations are as below:

HumanAdcy1-C2, *Homo sapiens* adenylate cyclase 1 second catalytic domain; Rat, *Rattus norvegicus*; Mouse, *Mus musculus*; Mytu, *Mycobacterium turberculosis*; Anabaena, *Anabaena* sp. PCC7120; Plasmodium, *Plasmodium falciparum*; Dicttyostelium, *Dictyostelium discoideum*; Treponema, *Treponema pallidum*; Streptomyces, *Streptomyces coelicolor*; Trypnosoma, *Trypnosoma brucei*; Leishmania, *Leishmania donovani*; Sc, *S.cerevisiae*; An, *A. nidulans*; Pb, *P. brasiliensis*; Sp, *S. pombe.*

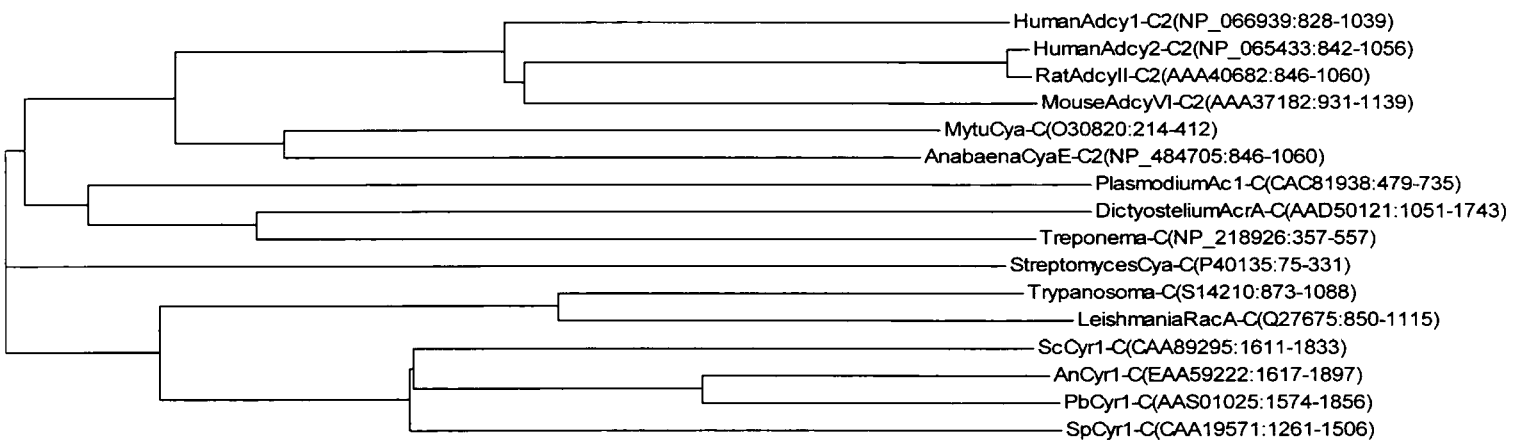
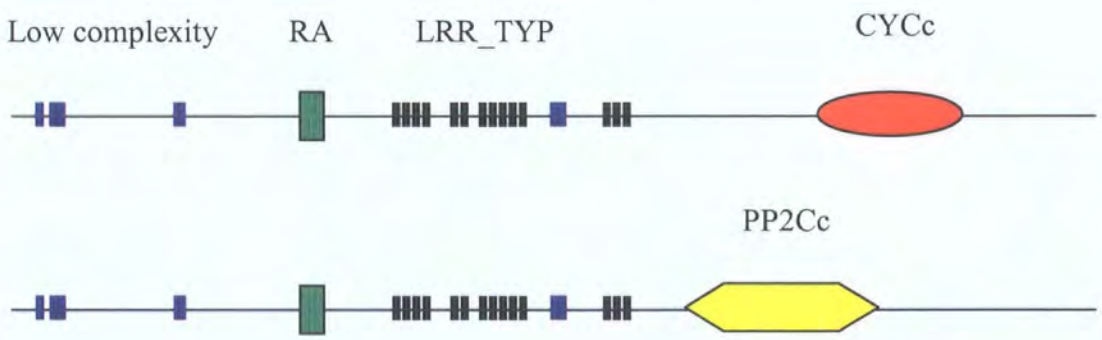


Figure 3.3-6. Domains of *Pb* adenylate cyclase PbCyr1 protein

Domain Name	Positions
Low complexity	39-50
Low complexity	57-78
Low complexity	337-354
RA	562-643
LRR_TYP	752-774
LRR_TYP	775-797
LRR	798-817
LRR_TYP	821-843
LRR_TYP	867-888
LRR_TYP	889-911
LRR_TYP	931-954
LRR_TYP	955-976
LRR_TYP	978-1000
LRR_TYP	1001-1024
LRR_TYP	1025-1047
Low complxity	1086-1108
LRR_TYP	1173-1196
LRR_TYP	1200-1220
LRR_TYP	1222-1244
PP2Cc	1341-1627
CYCc	1574-1856



Note: This figure is adapted from EMBL's SMART output when PbCyr1 was analysed. Due to the overlap in domains, there are 2 representations of the protein.

3.4 Differential Splicing at the First Intron of *PbCYR1*

Interestingly, when the cDNA fragment cPbAC-R21SIVII-12 (Figure 3.2-1) was amplified during the cloning of *PbCYR1* cDNA using SSP-PCR, 2 additional types of cDNA clones were obtained and named cPbAC-R20SIVI-8 and cPbAC-R21SIVI-2. The differences in cDNA sequence were found to be caused by differential types of splicing at the first intron after the genomic DNA sequencing was finished. Therefore, 3 versions of cDNA were supposed to exist due to differential splicing and they were accordingly named *PbCYR1*-cDNA-R21SIVII-12, *PbCYR1*-cDNA-R20SIVI-8 and *PbCYR1*-cDNA-R21SIVI-2 (Figure 3.4-1).

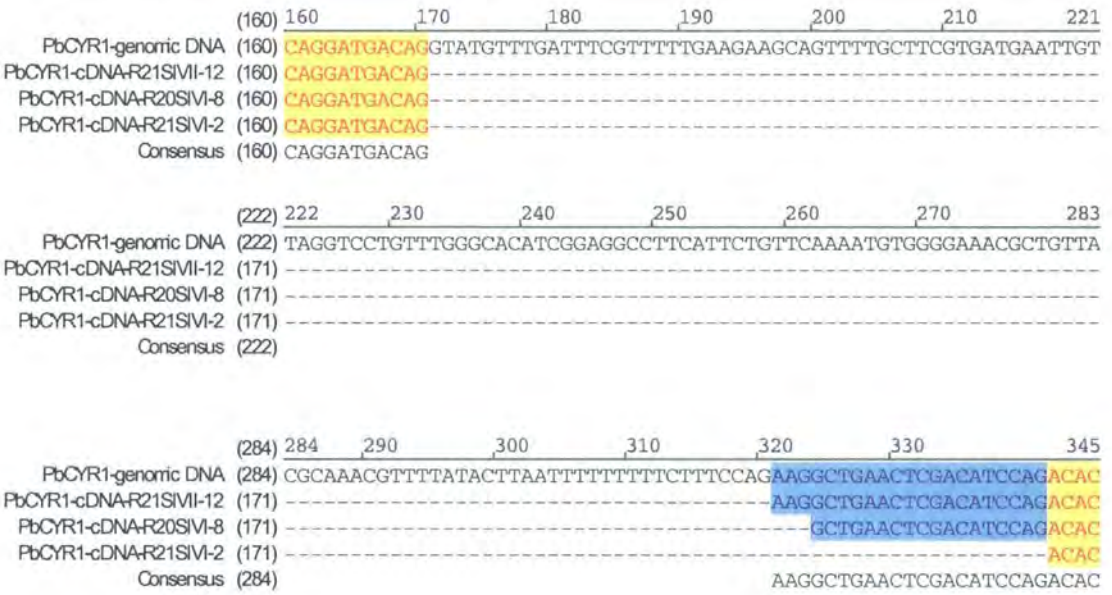
In order to check if there are further types of differential splicing and if there are additional introns, full-length cDNA cPbAC-F17R32 was amplified in a single RT-PCR using ABgene's Extensor Hi-Fedelity PCR kit with primer PbAC-F17 (positions -29-1) and PbAC-R32 (positions 7332-7364). The RT-PCR amplification of cPbAC-F17R32 was very successful because a single 6.6 kb band was obtained (Figure 3.4-2). Sequencing of cPbAC-F17R32 indicated that the cDNA was homogeneous except in the regions of the 1st and the 2nd intron, suggesting differential splicing in those regions. To delineate the 1st and the 2nd intron, cPbAC-F17R32 was cloned into pGEM-T Easy vector and 8 individual clones were submitted to sequencing analysis. Two new types of splicing were found. However, they were proved to be odd mis-splicing as indicated by the appearance of premature stop codons immediately following the spliced sites (Figure 3.4-3). The result is illustrated as below:

Clones	Description
4 clones of cPbAC-F17R32	The same as the sequence of PbCYR1-cDNA-R21SIVII-12 (Figure 3.4-1)
1 clone of cPbAC-F17R32	The same as the sequence of PbCYR1-cDNA-R21SIVI-2 (Figure 3.4-1)
PbCYR1-cDNA-F17R32-12	Mis-splicing (see Figure 3.4-3)
PbCYR1-cDNA-F17R32-16	Mis-splicing (see Figure 3.4-3)
PbCYR1-cDNA-F17R32-11	Deletion caused by secondary structure of mRNA during RT

In general, apart from the 2 types of mis-splicing, there are 3 types of sensible splicing in *PbCYR1* (Figure 3.4-1) at the 1st intron. They were named *PbCYR1* (full length cDNA version), *PbCYR1* (-3 bp cDNA version) and *PbCYR1* (-21 bp cDNA version), which in turn give rise to 3 types of proteins translated, i.e. full-length PbCyr1, truncated protein PbCyr1$^{\Delta 58}$ and PbCyr1$^{\Delta 58\text{-}64}$.

Figure 3.4-1 Three types of differential splicing at the first intron of *PbCYR1*. Nucleotide sequence and amino acid sequence alignment was done with Vector NTI 6.0 (Informax).

A: Three different types of splicing at first intron. They are *PbCYR1* full length cDNA version represented by clone PbCYR1-cDNA-R21SIVII-12 (171-320 spliced); *PbCYR1* -3 bp cDNA version represented by clone PbCYR1-cDNA-R20SIVI-8 (171-323 spliced); and *PbCYR1* -21 bp cDNA version represented by clone PbCYR1-cDNA-R21SIVI-2 (171-341 spliced).



B: Differential splicing leads to the 2 types of truncations for PbCyr1 protein, i.e. PbCyr1$^{\Delta 58}$ and PbCyr1$^{\Delta 58\text{-}64}$.

Figure 3.4-2 Full-length *PbCYR1* cDNA amplification by RT-PCR. RT-PCR was done by using primer PbAC-F17 (positions -29-1) and PbAC-R32 (positions 7332-7364). A single band of 6.6 kb cDNA was obtained. Further analysis of the band indicated differential splicings at the 1st and 2nd introns.

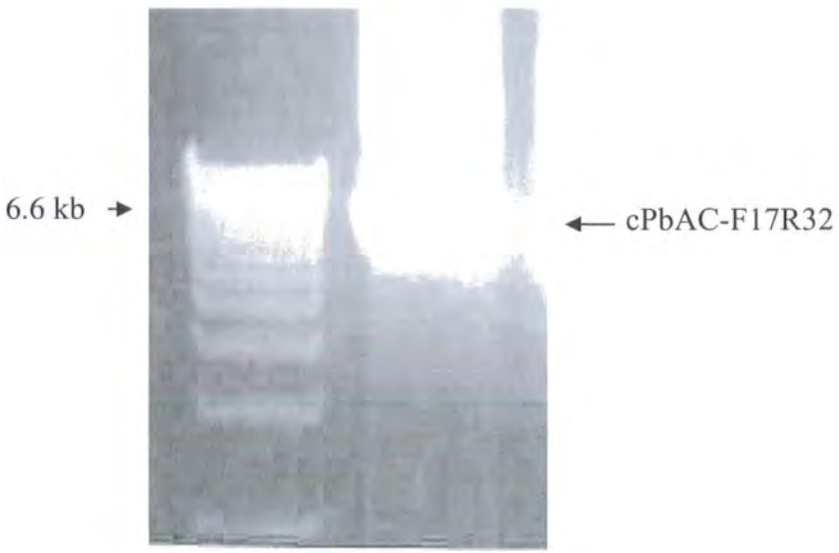
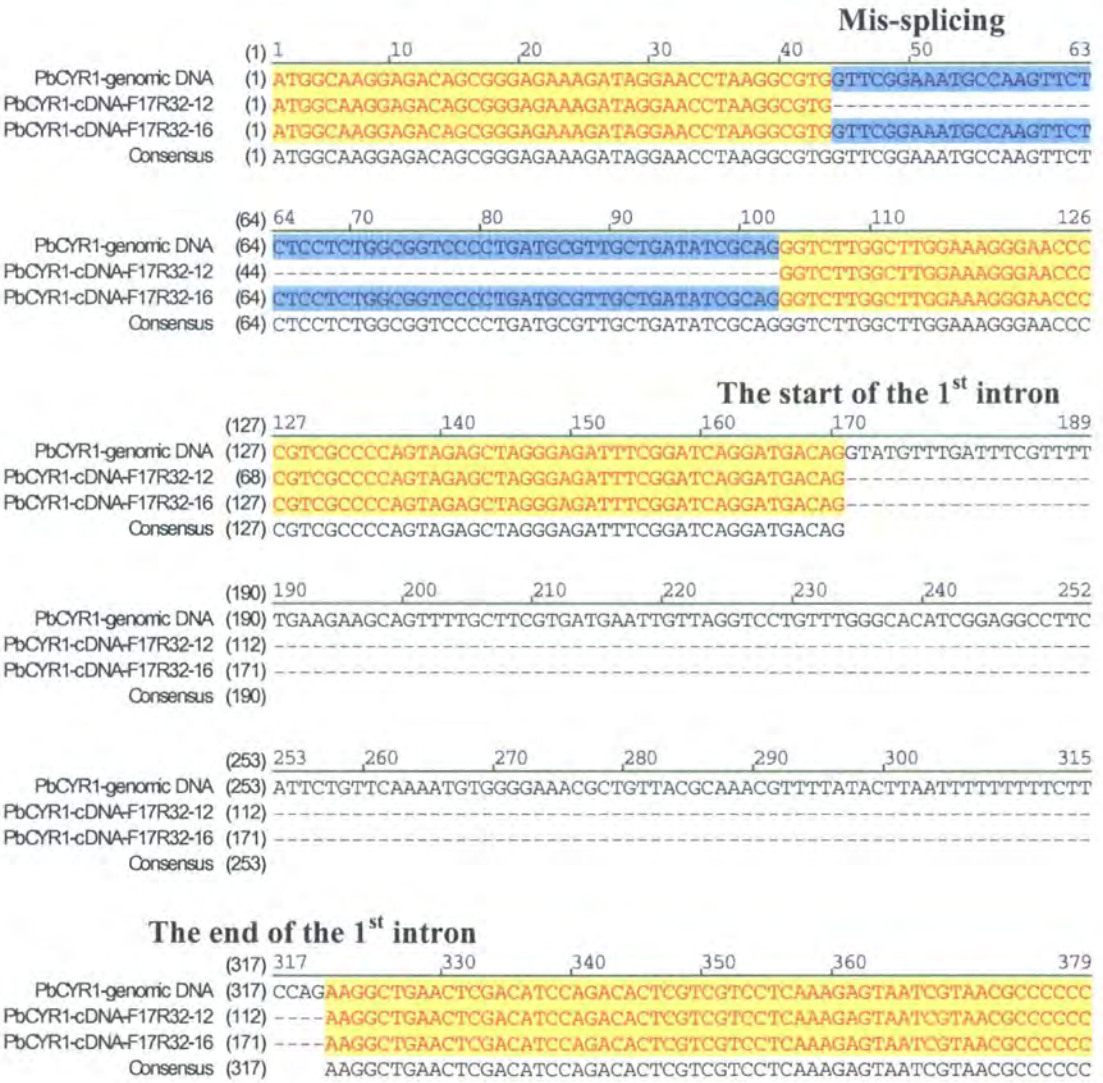
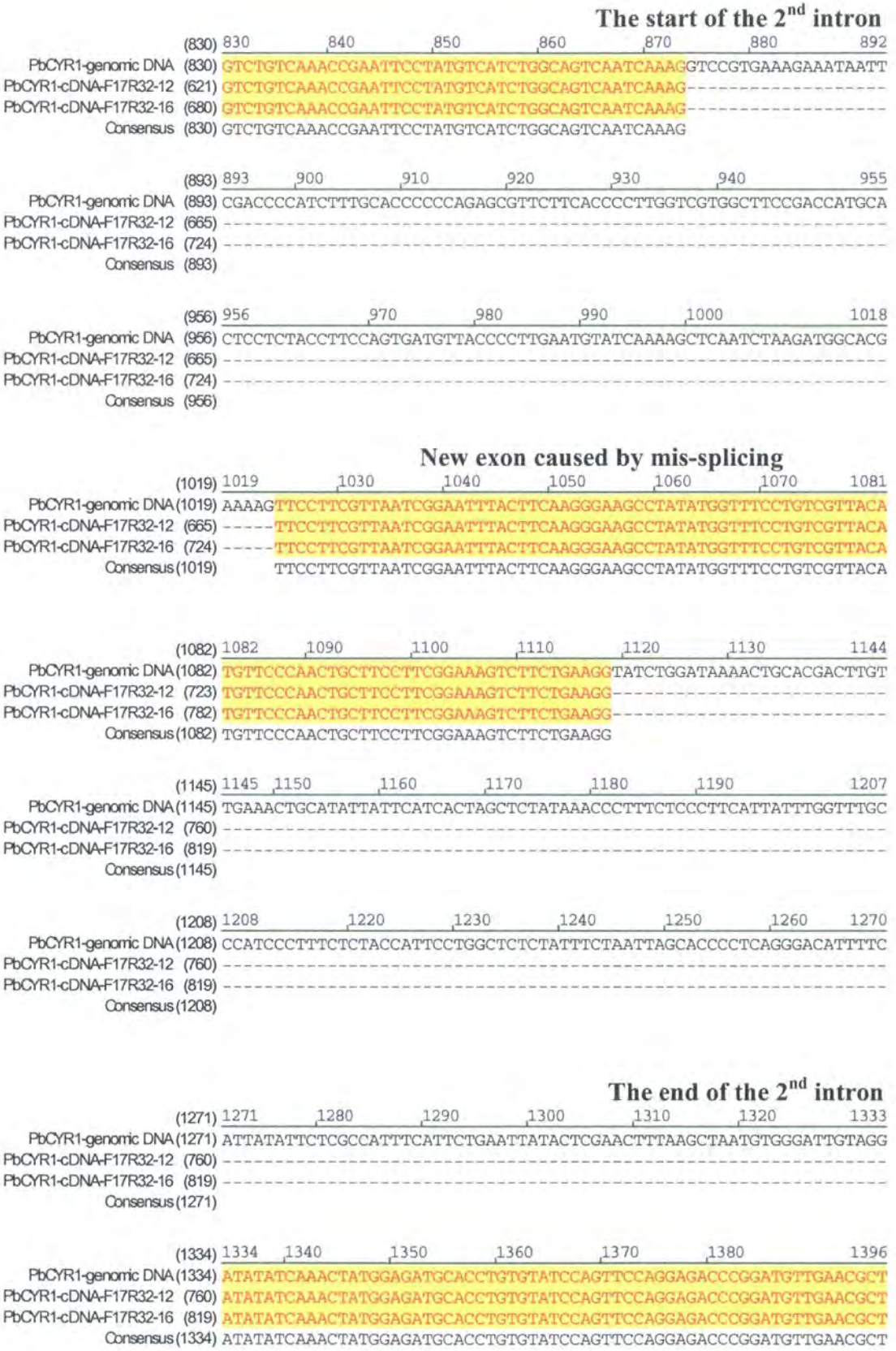


Figure 3.4-3 Illustration of 2 types of mis-splicing of *PbCYR1*. Nucleotide sequence alignment was done by using VectorNTI 6.0 (Informax). Two types of mis-splicing were observed when individual clones of RT-PCR products obtained with primer PbAC-F17 and PbAC-R32 were sequenced. Clones were considered to be mis-spliced because premature stop codons were found immediately after the mis-spliced site.

For clone PbCYR1-cDNA-F17R32-12, the mis-splicing (positons 45-102) happened before the first intron (positions171-320).

For clone PbCYR1-cDNA-F17R32-16, the 2nd intron (positions 874-1333) was turned into 2 sub-introns (874-1023 and 1119 to 1333), leading to premature stop codons in the new exon (1024-1018) such caused.




The start of the 2nd intron
PbCYR1-genomic DNA (830) GTCTGTCAAACCGAATTCCTATGTCATCTGGCAGTCAATCAAAGGTCCGTGAAAGAAATAATT
PbCYR1-cDNA-F17R32-12 (621) GTCTGTCAAACCGAATTCCTATGTCATCTGGCAGTCAATCAAAG
PbCYR1-cDNA-F17R32-16 (680) GTCTGTCAAACCGAATTCCTATGTCATCTGGCAGTCAATCAAAG
Consensus (830) GTCTGTCAAACCGAATTCCTATGTCATCTGGCAGTCAATCAAAG
PbCYR1-genomic DNA (893) CGACCCCATCTTTGCACCCCCCAGAGCGTTCTTCACCCCTTGGTCGTGGCTTCCGACCATGCA
PbCYR1-cDNA-F17R32-12 (665)
PbCYR1-cDNA-F17R32-16 (724)
Consensus (893)
PbCYR1-genomic DNA (956) CTCCTCTACCTTCCAGTGATGTTACCCCTTGAATGTATCAAAAGCTCAATCTAAGATGGCACG
PbCYR1-cDNA-F17R32-12 (665)
PbCYR1-cDNA-F17R32-16 (724)
Consensus (956)
New exon caused by mis-splicing
PbCYR1-genomic DNA (1019) AAAAGTTCCTTCGTTAATCGGAATTTACTTCAAGGGAAGCCTATATGGTTTCCTGTCGTTACA
PbCYR1-cDNA-F17R32-12 (665) -----TTCCTTCGTTAATCGGAATTTACTTCAAGGGAAGCCTATATGGTTTCCTGTCGTTACA
PbCYR1-cDNA-F17R32-16 (724) -----TTCCTTCGTTAATCGGAATTTACTTCAAGGGAAGCCTATATGGTTTCCTGTCGTTACA
Consensus (1019) TTCCTTCGTTAATCGGAATTTACTTCAAGGGAAGCCTATATGGTTTCCTGTCGTTACA
PbCYR1-genomic DNA (1082) TGTTCCCAACTGCTTCCTTCGGAAAGTCTTCTGAAGGTATCTGGATAAAACTGCACGACTTGT
PbCYR1-cDNA-F17R32-12 (723) TGTTCCCAACTGCTTCCTTCGGAAAGTCTTCTGAAGG
PbCYR1-cDNA-F17R32-16 (782) TGTTCCCAACTGCTTCCTTCGGAAAGTCTTCTGAAGG
Consensus (1082) TGTTCCCAACTGCTTCCTTCGGAAAGTCTTCTGAAGG
PbCYR1-genomic DNA (1145) TGAAACTGCATATTATTCATCACTAGCTCTATAAACCCTTTCTCCCTTCATTATTTGGTTTGC
PbCYR1-cDNA-F17R32-12 (760)
PbCYR1-cDNA-F17R32-16 (819)
Consensus (1145)
PbCYR1-genomic DNA (1208) CCATCCCTTTCTCTACCATTCCTGGCTCTCTATTTCTAATTAGCACCCCTCAGGGACATTTTC
PbCYR1-cDNA-F17R32-12 (760)
PbCYR1-cDNA-F17R32-16 (819)
Consensus (1208)
The end of the 2nd intron
PbCYR1-genomic DNA (1271) ATTATATTCTCGCCATTTCATTCTGAATTATACTCGAACTTTAAGCTAATGTGGGATTGTAGG
PbCYR1-cDNA-F17R32-12 (760)
PbCYR1-cDNA-F17R32-16 (819)
Consensus (1271)
PbCYR1-genomic DNA (1334) ATATATCAAACTATGGAGATGCACCTGTGTATCCAGTTCCAGGAGACCCGGATGTTGAACGCT
PbCYR1-cDNA-F17R32-12 (760) ATATATCAAACTATGGAGATGCACCTGTGTATCCAGTTCCAGGAGACCCGGATGTTGAACGCT
PbCYR1-cDNA-F17R32-16 (819) ATATATCAAACTATGGAGATGCACCTGTGTATCCAGTTCCAGGAGACCCGGATGTTGAACGCT
Consensus (1334) ATATATCAAACTATGGAGATGCACCTGTGTATCCAGTTCCAGGAGACCCGGATGTTGAACGCT

3.5 Construction of a High Fidelity *PbCYR1* cDNA

In order to obtain a 6.3 kb high fidelity cDNA for *PbCYR1*, small segments of the *PbCYR1* cDNA were cloned and sequenced to confirm their fidelity at the amino acid level. These segments of cDNA were then recombined into a full-length cDNA. The procedures for this construction are illustrated in Figure 3.5-1 and Figure 3.5-2. In general, 3 important constructions were obtained, namely pYES6-cPbAC-F26R34-1-14, pBSK-cPbAC-F26R34-1 and pGEMTE-cPbAC-F26R34-1. pYES6-cPbAC-F26R34-1-14 was constructed in pYES6/CT (Invitrogen) using the -21 bp cDNA version of *PbCYR1*, with a point mutation that introduced a single amino acid change in the protein derivative (Q600R; nucleotide A at position 2,408 in Figure 3.3-1 replaced by G in addition to A2S introduced by primer PbAC-F26). The pBSK-cPbAC-F26R34-1 and pGEMTE-cPbAC-F26R34-1 constructions were made with full-length *PbCYR1* cDNA, into which no point mutation at the amino acid level had been introduced (except for A2S introduced by primer PbAC-F26), cloned into pBSK (Stratagene) and pGEM T Easy (Promega) vectors respectively (pGEM T Easy vector was artificially introduced with a *Kpn*I site). These constructs were used as templates in sub-cloning of genes for yeast two-hybrid analyses or other downstream work.

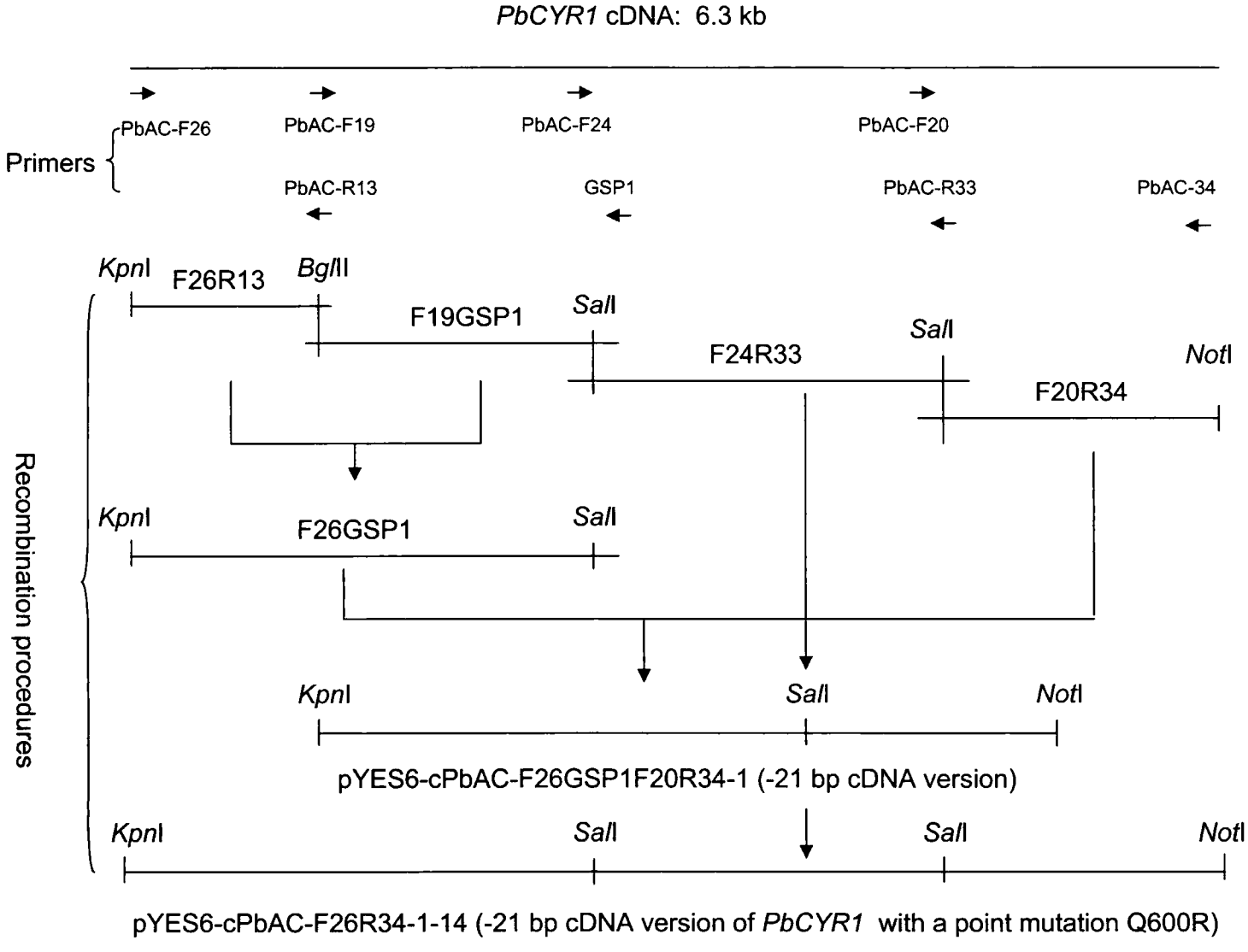


Figure 3.5-1 Construction of fidelity *PbCYR1* cDNA

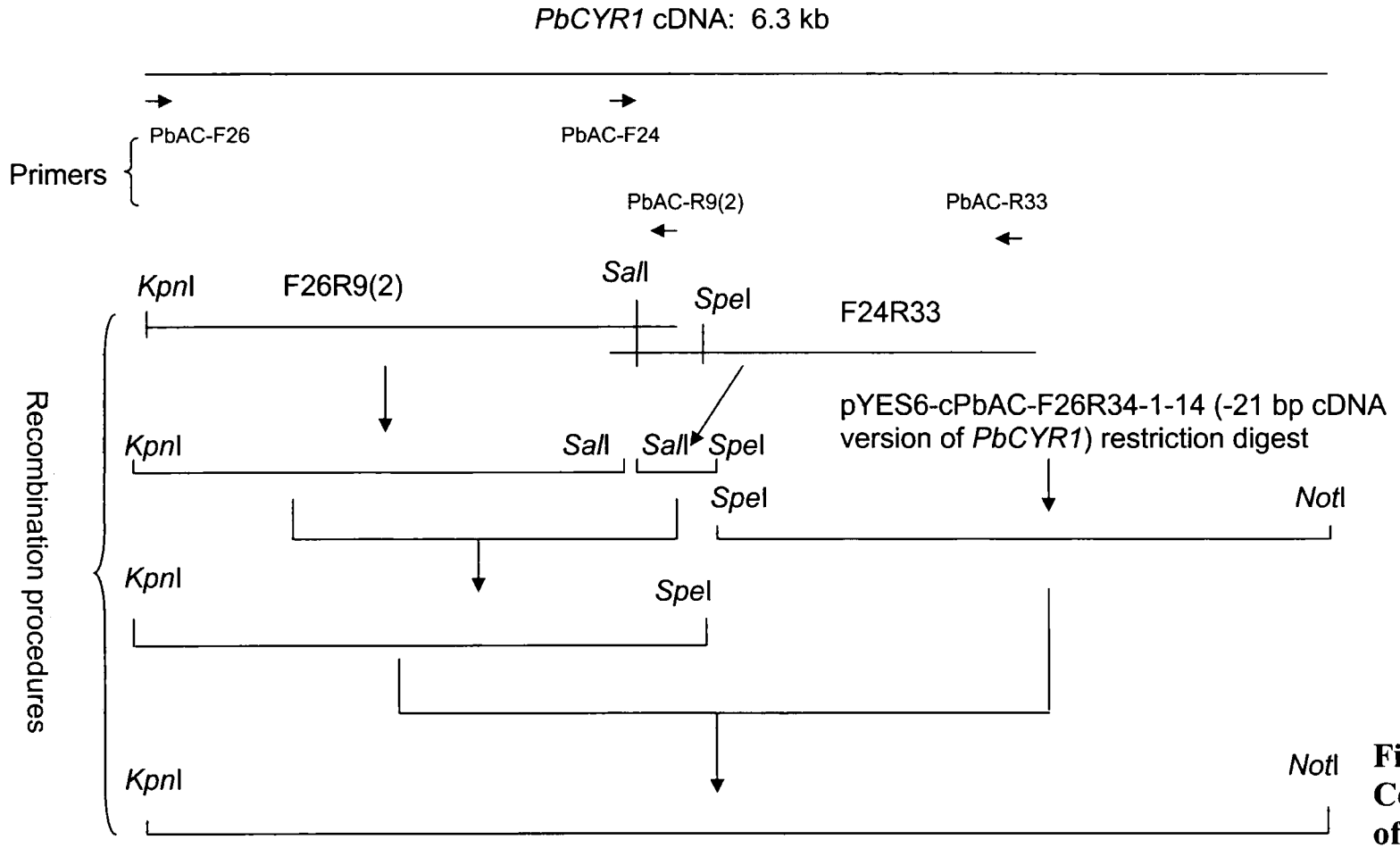


Figure 3.5-2 Construction of fidelity *PbCYR1* cDNA

pBSK-cPbAC-F26R34-1 and pGEMTE-cPbAC-F26R34-1
(both constructions are full-length cDNA version of *PbCYR1*)

3.6 Cloning of 3 Iso-forms of G Protein α Subunit Genes *PbGPA1, 2 and 3* from *Pb01*

The strategy for cloning of *PbGPA1-3* was PCR amplifications, with walking on both genomic DNA and cDNA. The cloning procedures are illustrated in Figure 3.6-2, Figure 3.6-3 and Figure 3.6-4. Primers were designed on the basis of immediate sequencing results. At the beginning, two degenerate primers, PbGPA-DGF5 and PbGPA-DGR2, were designed corresponding to conserved amino acid residues (AS)GESGKST and RKKWIHC, respectively. Three fragments of 578 bp, 680 bp and 644 bp were amplified from genomic DNA, and designated *PbGPA1*, *PbGPA2* and *PbGPA3* accordingly (Figure 3.6-1). The fragments were sequenced and specific primers were designed, with which, fragments upstream and downstream of the genes were amplified from both our genomic walker library and pDNR-*Pb01*cDNA library.

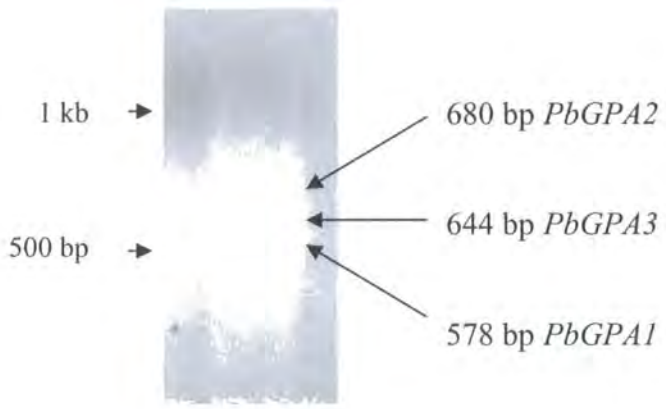
On the basis of the gPbGPA-DGF5R2 (578 bp, *PbGPA1*) sequence, two specific primers, PbGPA1-F1 and PbGPA1-R2, were designed for upstream and downstream SSP-PCR. By using these 2 specific primers and 2 anchor primers (M13R1 and M13F1), 2 cDNA fragments, namely cPbGPA1-F1M13R1 and cPbGPA1-R2M13F1, were amplified from the pDNR-*Pb01*cDNA library plasmids. An upstream genomic DNA fragment, gPbGPA1-R4AP21-3, was amplified by SSP-PCR using our GenomeWalker library. A downstream genomic DNA fragment, gPbGPA1-F2AP3-2, was amplified as well. The genomic DNA sequence was finished by amplifying a fragment, named gPbGPA1-F7R1, bridging gPbGPA1-R4AP21-3 and gPbGPA1-F2AP3-2. The full-length *PbGPA1* cDNA sequence was amplified with 2 specific primers, PbGPA1-ExGPAF1 and PbGPA1-pYER3, which include the ATG start

codon and TGA stop codon respectively. The cloning procedure for *PbGPA1* is illustrated in Figure 3.6-2.

On the basis of the gPbGPA-DGF5R2 (680 bp, *PbGPA2*) sequence, 4 specific primers, PbGPA2-F1, PbGPA2-F2, PbGPA2-R1 and PbGPA2-R2, were designed for cloning of *PbGPA2* by SSP-PCR. By using specific primers PbGPA2-F1 and PbGPA2-R1, and an anchor primer AP3, 2 genomic DNA fragments, namely gPbGPA2-F1AP3 and gPbGPA2-R1AP3, were amplified from our GemomeWalker library. Another 2 cDNA fragments were amplified, namely cPbGPA2-R2M13F1 and cPbGPA2-F2M13R1, from our pDNR-*Pb01*cDNA library. Then a genomic DNA fragment was amplified using specific primers PbGPA2-R2 and PbGPA2-F4 (designed from sequence of cPbGPA2-R2M13F1) and *Pb01* genomic DNA as target. A further upstream genomic DNA sequence, gPbGPA2-R8AP4-4, was amplified from our GenomeWalker library. The full-length cDNA of *PbGPA2*, namely cPbGPA2-F6R6, was amplified from *Pb01* mRNA with PbGPA2-F6 and PbGPA2-R6, which included the start and stop codons respectively. The cloning procedure for *PbGPA2* is also illustrated in Figure 3.6-3.

On the basis of the gPbGPA-DGF5R2 (644 bp, *PbGPA3*) sequence, two specific primers PbGPA3-F1 and PbGPA3-R1 were designed for cloning *PbGPA3* by SSP-PCR. By using the 2 specific primers and the anchor primer AP3, 2 genomic DNA fragments, gPbGPA3-F1AP3-2 and gPbGPA3-R1AP3-2, were amplified from our GenomeWalker library. A new primer PbGPA3-R4 was designed and used to amplify a further upstream genomic fragment, gPbGPA3-R4AP3-3. The full-length cDNA was deduced from the genomic DNA sequence. Two primers, PbGPA3-F5

Figure 3.6-1. Three iso-forms of G protein alpha subunit amplified by using degenerate PCR. With a pair of degenerate primer PbGPA-DGF5 and PbGPA-DGR2, 3 iso-forms of *PbGPA* were amplified from *Pb01* genomic DNA.



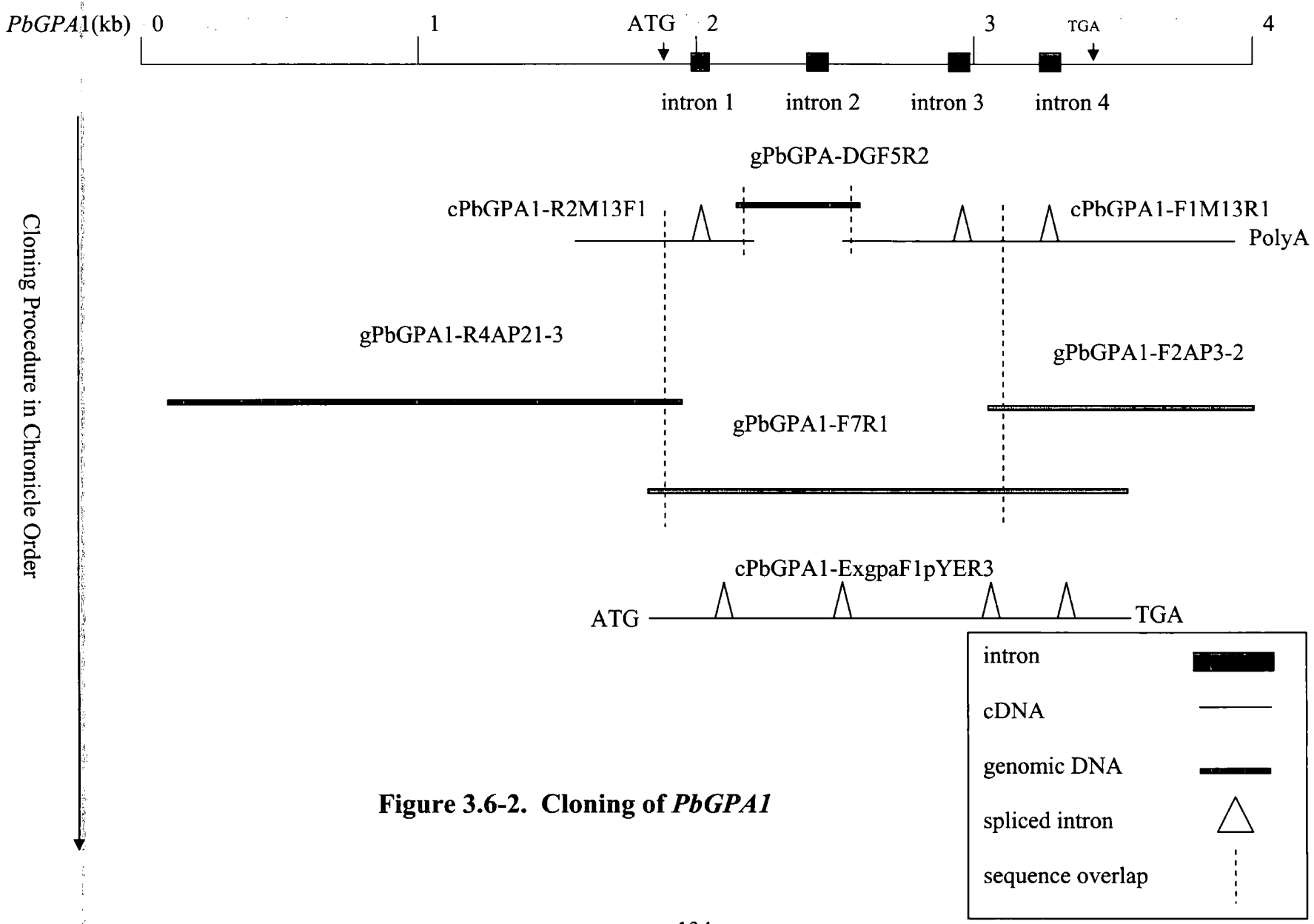


Figure 3.6-2. Cloning of *PbGPA1*

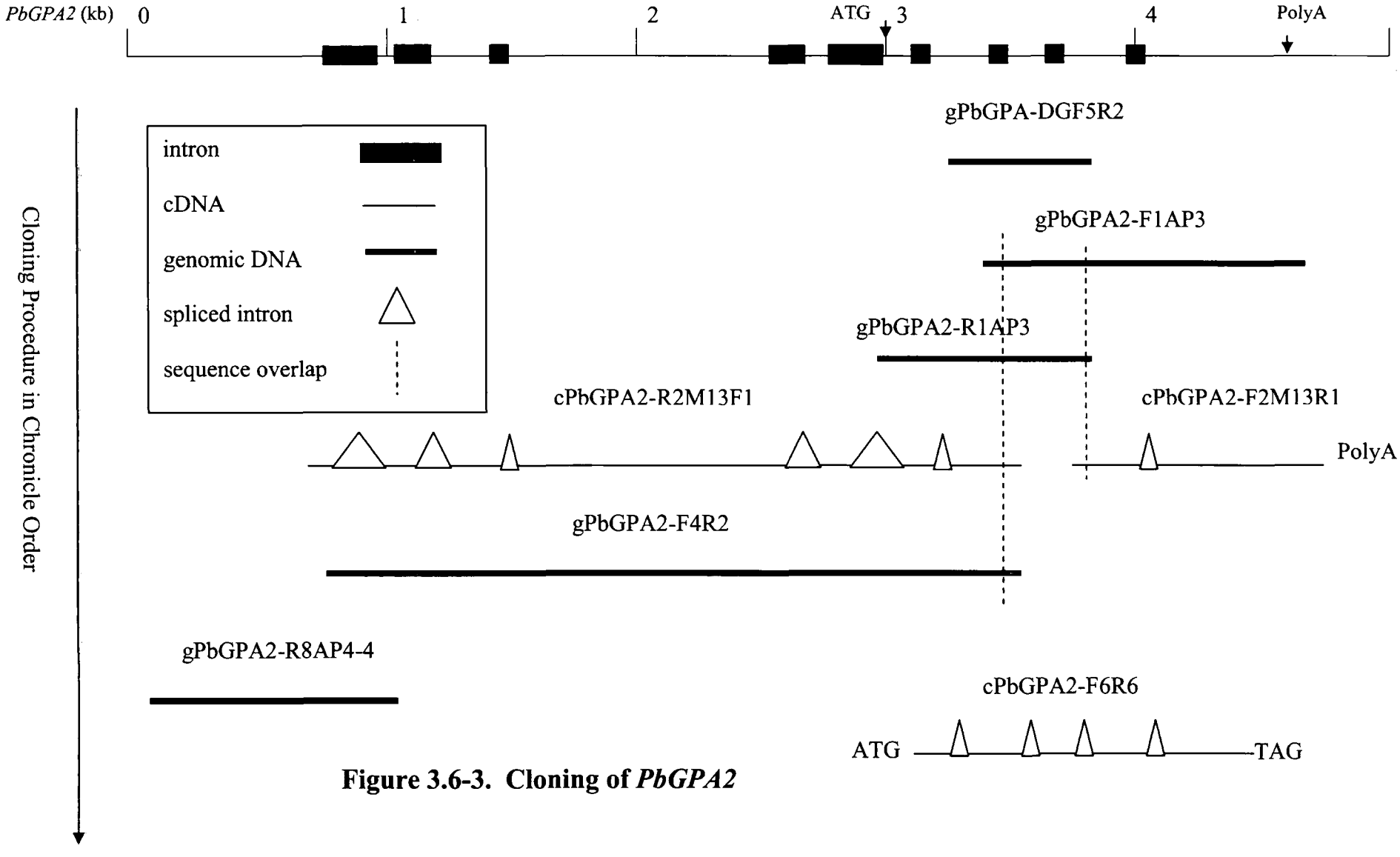


Figure 3.6-3. Cloning of *PbGPA2*

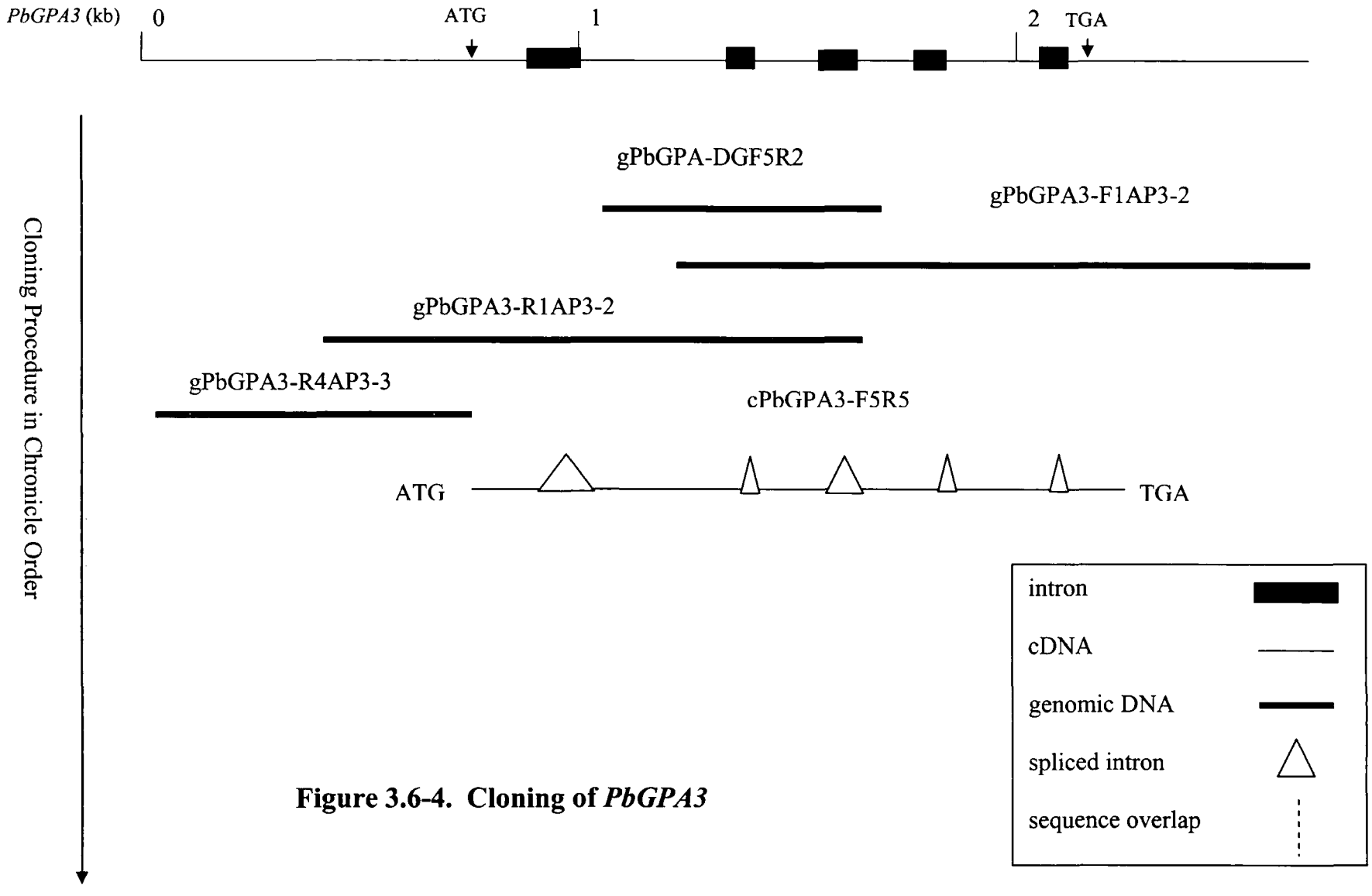


Figure 3.6-4. Cloning of *PbGPA3*

and PbGPA3-R5, including start and stop codons respectively, were designed and used to clone the full-length cDNA cPbGPA3-F5R5 from pDNR-*Pb01*cDNA library plasmids. The cloning procedure for *PbGPA3* is also illustrated in Figure 3.6-4.

3.7 The Nucleotide Sequence and the Deduced Amino Acid Sequence of G Protein α Subunit Genes *PbGPA1, 2 and 3*

Following the successful amplification of genomic DNA and cDNA fragments, the genomic DNA sequence, cDNA sequence and the deduced amino acid sequence of *PbGPA1, 2 and 3* were determined; and these are illustrated in Figure 3.7-1, Figure 3.7-2 and Figure 3.7-3. The *PbGPA1, 2 and 3* genes can now be retrieved from GenBank with accession numbers of AY550246, AY550248, and AY550247 respectively. The *PbGPA1* gene is interrupted by 4 introns. The coding sequence of *PbGPA1* gene is 1,059 bp long and encodes a protein of 353 amino acids with a molecular weight of 41 kDa and predicted pI of 5.0 (Figure 3.7-1). The *PbGPA2* gene is interrupted by 4 introns. Interestingly, the cDNA of *PbGPA2* is quite long with an additional 5 introns spliced before the start codon ATG (Figure 3.7-2 and 3.6-3). The function of this long stretch of un-translated cDNA (about 1.5 kb) needs to be investigated further. The coding sequence of *PbGPA2* is 1,077 bp long and encodes a protein of 359 amino acids with a molecular weight of 41 kDa and predicted pI of 6.2 (Figure 3.7-2). The *PbGPA3* gene is interrupted by 5 introns. The coding sequence of *PbGPA3* is 1,068 bp long and encodes a protein of 356 amino acids with molecular weight of 41 kDa and predicted pI of 6.3 (Figure 3.7-3).

The PbGpa1, 2 and 3 proteins are conserved within themselves; with PbGpa1 and PbGpa2 displaying 43% identity; PbGpa2 and PbGpa3 41% identity; and PbGpa1

and PbGpa3 47% identity. Interestingly, the 3 genes share some intron-splicing sites exactly at the same positions in amino acid sequence, indicating that they were created by duplication during evolution (Figure 3.7-4).

The currently known fungal G protein α subunits can be grouped into 3 distinct subfamilies (Figure 3.7-5). The close relatives of PbGpa1 are as the follows:

Protein: organism	**% identity to PbGpa1**
PmGasA: *P. marneffei*	93%
AnFadA: *A. nidulans*	92%
NcGna1: *Neurospora crassa*	91%
MgMagB: *Magnaporthe grisea*	91%
CpCpg1: *Cryphonectria parasitica*	92%
UmGpa1: *Ustilago maydis*	75%
CnGpaB: *Cryptococcus neoformans*	62%
ScGpa1: *S. cerevisiae*	37%

The close relatives of PbGpa2 are as below:

Protein: organism	**% identity to PbGpa2**
AnGanA: *A. nidulans*	78%
PmGasB: *P. marneffei*	78%
MgMagC: *Magnaporthe grisea*	52%
CpCpg3: *Cryphonectria parasitica*	51%
NcGna2: *Neurospora crassa*	52%
UmGpa2: *Ustilago maydis*	47%
SpGpa1: *S. pombe*	39%

The close relatives of PbGpa3 are as below:

Protein: organism	**% identity to PbGpa3**
PmGasC: *P. marneffei*	86%
AnGanB: *A. nidulans*	85%
NcGna3: *Neurospora crassa*	78%
MgMagA: *Magnaporthe grisea*	77%
CpCpg2: *Cryphonectria parasitica*	76%
UmGpa3: *Ustilago maydis*	68%
CnGpa1: *Cryptococcus neoformans*	66%
ScGpa2: *S. cerevisiae*	50%
SpGpa2: *S. pombe*	45%

Figure 3.7-1 The nucleotide sequence and the deduced amino acid sequence of *PbGPA1*

```
-1950   TTTAAGCTATACAGGGTTCAAGTTTCCTTCCAAGCCTTCTAGAAGCATCA
-1900   CGATTGCACTGGGCTCCACAAGTGAAGAGGGGGTGGAAACGAAAGGCTGG
-1850   TAAATTTGGGATTTTTGTAGGGAATGGCTTGGAGGAGTTTAACCCTAGGC
-1800   GAACGGAACAATTATTTTTGGAAGGGAATGGCTTGTCTATGACTCGTGGG
-1750   TTTTCGCCTCTTGTTCTGGGCTGTTGAACTGGGATGATGAGGATGACATG
-1700   CAAGGTAGACTTGTTCTCTAATATCTCCTCCTGTGGAGATATAAATTTCT
-1650   GATTAAATTCTCCCAATTTCTCCCAATTTCTTCCGTTTTTCGTTTTCCCC
-1600   TCTGGCGGGATTGGGTTCCACTCTGCAGTCAAGCTGCCGATTCGTCAGCG
-1550   GGATAAACATGGGTGCAAGAACCATTTCAACCTTTTTTCCGTTTATCTCT
-1500   AAAGCCCGACTTTGGGAAGTTTCTTCTATTTTCGGGACACTGCGTCCTCT
-1450   ATTTATCGATGTTGATACGCTGGGAACAATGGATGCCACGTTTGCCTGAG
-1400   CTTACCTAGGCAGAAAGGTATCCAACGGGCAAATTCTTCTATCTTTCTCG
-1350   AGAAATTTCGGGACGTATTTCTAAAGGGGCACACAAACGCAGGCAGACTG
-1300   CAGTGCAGTATTATCTCCAAGGCTGGGAGTGGATTAATAATTTGATACCC
-1250   AAGAAACTAATAATGTCATCCTAATAAGCCATGGGTAATTGCGCCCGTTA
-1150   TAGTTACCTCTCCTCCCCCTTTATTTTATTTCTTACACGAGATGGGGGAC
-1100   TTGTAAGGAAGAGACCAATTCTCTGGGCGGTCGGTGGGTGGGTATTCGGG
-1050   GTGTTCTGTTGTTACGAAGCAGGTCACCAGCGGACTCATTCCAACCAACT
-1000   CGGCCATGGATGGAGGAGAAACCTGCCCCTTGTACTGCACATACTAGTGT
 -950   ATTTAGCACTTGACACCTCGTTTGGCGCTGGACCTTGGCGTGTGTTGCAA
 -900   GGCGGTTGAATATTGTATGTATCATAACTTATTGAACTGTAACATAACAG
 -850   CCGTAAAGGCCGTGACCATACCGTACAACGCTTCCTCTTCCTTCGTCCCA
 -800   CCTTCCCCTTCTCAGCCGGCATACCCTTGTTGTGTTCTTTCCTGCTTCTT
 -750   CCTCCTCCATTGTTCTTTGCCCAGGCCAACCTCCAAACCTCCAAACCTCC
 -700   AACCTCCAACCTCCTCCTTCGCTATCTTCATCTCCATCTTCACCTTCAAT
 -650   CCTCTAATCTCCCGCCCTGCCGTGTTGTTGGACTGTTGGCCTGAACCTGT
 -600   AGGATTCCATTCCAGTCCTTTCCCGCAAAACCCAGCCAGTCTTCAGCAAA
 -550   TTGAATCGCTCTTTTCCCAGCGTGCGATTTGGTCCGCTCTCCCCTAAGTT
 -500   CTCTTTGACTCCCTGCAGACCACCACTACCTGACCTCTTCTGCTCTGCCC
 -450   ACCAACCCTCTCTCTTTGGAATACGGTATTATCGGTATCTCCAAAGCAAT
 -400   ACTGAATTGAGTTGATTGGTCTTGGTTTAATTGTTTCTTTCCCCCATTGG
 -350   AGTATATTGTGAGTGTAGAATGTCTTGACTTTATGGCTTGCCTCTTGGGC
 -300   TCTTGTTGCCGCATTGTCGGGACGACGGCATTGGGGGAATGTGACCGGCT
 -250   GCAACCCTTTTGCTGGCCAGCAGCACCAATCATCTTCTACCAATCGATTC
 -200   ACTCTCTCTTTACCGCCCTACCTGCTTTTTTTTACCTTGTCGAGCTCTGG
 -150   GATGTGTAGTTGATCCATTTCCCACTGACATGAAGCAGAACCTGTACATT
 -100   CCGATATCGTCCAGAGCATTTTGTGTCATTCATCCATCTTGCTTTACGCC
  -50   CTGAAAAATAAACTCACCCCAGTCTTTACACATTCTGTAACTTGACAACA

    1   ATGGGGTGTGGAATGAGCACCGAGGACAAGGAAGGAAAGGCTAGAAATGA
    1     M  G  C  G  M  S  T  E  D  K  E  G  K  A  R  N
   51   AGAGATTGAGAATCAACTGAAGAGGGATAAGATGCTGCAGAGAAACGAGA
   17   E  E  I  E  N  Q  L  K  R  D  K  M  L  Q  R  N  E
  101   TCAAAATGCTCTTGCTTGgtaagatgattctttttccttttgtttaatcc
   34    I  K  M  L  L  L
  151   ggctgtatgacttacttggtctgaaccacccagGTGCTGGAGAGTCTGGC
   40                                    G  A  G  E  S  G
  201   AAATCCACAATTTTGAAACAGATGAAACTCATTCACGAGGGTTCATATTC
   46     K  S  T  I  L  K  Q  M  K  L  I  H  E  G  S  Y
  251   GCGAGATGAGAGAGAATCCTTCAAGGAAATCATTTTCAGCAACACCGTCC
   62   S  R  D  E  R  E  S  F  K  E  I  I  F  S  N  T  V
  301   AGTCGATGCGCGTTATTCTCGAGGCTATGGAAGGACTAGATATCCCGTTG
   79    Q  S  M  R  V  I  L  E  A  M  E  G  L  D  I  P  L
  351   GATGACCAGCGTGCCGAATACCATGTTCAGACTATCTTCATGCAACCAGC
   96     D  D  Q  R  A  E  Y  H  V  Q  T  I  F  M  Q  P
  401   ACAGATTGAAGGCGATTGCTTACCCCCCGATGTTGGCAGTGCAATCGACG
  112   A  Q  I  E  G  D  C  L  P  P  D  V  G  S  A  I  D
  451   CCCTTTGGAACGATGCTGGTGTACAAGAATGTTTCCGGAGATCTCGCGAG
  129    A  L  W  N  D  A  G  V  Q  E  C  F  R  R  S  R  E
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501   TATCAGCTGAATGACTCTGCACGATAgtaagcttcattgctgccacaaac
146     Y  Q  L  N  D  S  A  R
551   acaatatatttgattaataaccaagatatatagCTATTTTGACTCCATCA
154                                     Y  Y  F  D  S  I
601   AGCGTATCGCCGCATCCGATTATTTACCTAGCGACCAGGATGTCCTTCGC
160    K  R  I  A  A  S  D  Y  L  P  S  D  Q  D  V  L  R
651   TCCCGTGTTAAGACCACAGGTATCACGGAGACAACGTTTATCATTGGCGA
177     S  R  V  K  T  T  G  I  T  E  T  T  F  I  I  G
701   TCTCACGTACCGTATGTTCGACGTAGGTGGTCAGCGTTCTGAGCGCAAGA
193   D  L  T  Y  R  M  F  D  V  G  G  Q  R  S  E  R  K
751   AGTGGATCCATTGCTTTGAAAACGTCACAACCATTCTTTTCCTCGTTGCT
210    K  W  I  H  C  F  E  N  V  T  T  I  L  F  L  V  A
801   ATCTCTGAGTACGACCAGCTCCTGTTCGAAGACGAAACCGTCAACCGTAT
227     I  S  E  Y  D  Q  L  L  F  E  D  E  T  V  N  R
851   GCAGGAAGCTCTCACACTTTTCGATTCCATTTGCAATTCGAGATGGTTTA
243   M  Q  E  A  L  T  L  F  D  S  I  C  N  S  R  W  F
901   CCAAGACTTCTATCATCCTCTTTCTCAACAAGATTGATCGGTTTAAGGAG
260    T  K  T  S  I  I  L  F  L  N  K  I  D  R  F  K  E
951   AAACTGCCTGTTAGCCCAATGAAAAACTACTTCCCTGATTACGAAGgttt
277     K  L  P  V  S  P  M  K  N  Y  F  P  D  Y  E
1001  gttttcgcccaactcctttatttttctcttattcatgataaacgttgctg

1051  atattgtgtaccagGGGGTGCCGAATACACTGCTGCTTGCGACTATATCC
292                  G  G  A  E  Y  T  A  A  C  D  Y  I
1101  TCAACCGCTTCGTCTCGCTCAACCAGGCTGAGCAGAAGCAAATTTATACA
304    L  N  R  F  V  S  L  N  Q  A  E  Q  K  Q  I  Y  T
1151  CACTTCACCTGTGCTACTGATACCTCTCAAATCCGATTCGTCATGATGGC
321     H  F  T  C  A  T  D  T  S  Q  I  R  F  V  M  M
1201  TGTCAACGgtatgtggcttgttcatttttatgtacaaattaatactaga
337   A  V  N
1251  atcatacatgtcatgctaactctagagtccagATATAATCATCCAGGACA
340                                     D  I  I  I  Q  D
1301  ACCTTCGCCTCTGTGGACTGATATGACGAAGTCTTTTGAATACGTTTGAT
346    N  L  R  L  C  G  L  I  *
1351  TCTTTTTAGTTACTTTTGATTTTTTTCGCTAATTAGATAATTTTCCTTTT

1401  CACGCCATGTGTATTTGATATTACCTTCAGCTTTTTCTTTGTACCTTGTC
1451  TTCCTCTTGGTTAAGGTTTATCTCCGGCCCGCTCCTCCTCCCTGACCTGT
1501  CAGCATCTGTCATTCAGAATTTCCTTTTCTATTAAGACATCTATACTTTT
1551  GCCCATATTATGGAGGATTTGACCAGTGGTTGAAAAGCCCTTTTTCCTAC
1601  ATCCCAAAAAACATGGAGTAATGCAGCCTCGTCAGCACAACCGGCACCACA
1651  TGAACGGGACATACGATTTCTTTCCCTGACCACCCTTTCCTTATGTGTCT
1701  CTTTTTTCTTCAGATCCATCTTGCCTGATCTTGTTACTTTTGTTTTTTTC
1751  AATTACCCTGCATGGTTTCCTTCTCAGTTTGATTTGAGATTATTTCTCTT
1801  TTTTCACCCTTTTTTATATCCTCTTGATGTATCTGTTGTGCGTATGAGGT
1851  ATGTTTTTTTTTCCATACCTTATTTTTATTCTTTTTGTTAGGGATTCTTT
                                        Polyadenylation site
1901  TCTCTTTTCTTTTTCATTTG
```

Notes:

Nucleotide positions are written in red numerals, amino acids in blue.

Introns are denoted by lowercase letters with red color.

Polyadenylation site is underlined.

Figure 3.7-2 The nucleotide sequence and the deduced amino acid sequence of *PbGPA2*

```
-3000   TTGAGAAATATAATTGCTAAGTATAAGNTTTTATTAAAAGATATATACAA
-2950   CTTTGATAAAATTGAACNTGCTATGAAAGTTTATATCATTGCTAAAAATA
-2900   TTACTAGTAGTGAGTGCTACNGTAGAGCAAAAGCTTTGATCAAATTATTA
-2850   AAATTTGTGAATATAAAAATGATCAGTATTATTATTTATGAAAAAGTAAT
-2800   ATCNGAATATATTCNATATAAATAAAATAAAAAAGTAAAAATGCNAGAGA
-2750   TCTAAATATCAAATTTAACNAAAAATAGAAGATTTAACTAGTAAAGAAGT
-2700   TCAAATTCTTCTAGCTTTATCTGCATCATTTGCATCATTTGCAGCATTTG
-2650   TTGAACAGTCAATTATTCAATTTTATCAAGTTCTTATTACTTGCATTTGC
-2600   TTCATGCTGATGAGCTCTACAACAATGCAGCAATTGTAATATTNTTAGGC
-2550   ATACAAGATTAAGATGTTCTCAACTGTGTGTAATTTAATTTAATTTAATT
-2500   TAATTGAGTGTTGTAGCATTGAGATAATTTGAATTAAAATCTAATAAGCT
-2450   GAGCTCTGGTGATTTTGGTGTTGCACTCACTTGAGAGTTGTGCTCACTTG
-2400   GGAAATACATTAACTAACTAGTTATAGTTAGTTAACTAATAATTAACTAA
-2350   CTTTGGTCATTGTGTTCCGGATATTTCTTCTTGTGACCATTTAAAGAACA
-2300   AAGTCAATGAGTGCAAACGGGATCTGCAAGGGAGCGTTTAGTTCAAAAGC
-2250   AAAGGTCCACGGGCAATAAAATTATTACCAGCGCATGTCCCGTGGCATTT
-2200   GAAAAGTGCGAAGAAAACAACGAGACCCATAACTATATTAAATGCCATTA
-2150   TGCTTTGCAATGAATTCAGgcaagaaaattccatgcatatctatagacaa
-2100   ttcttcatcggacaacacactgaacagaggggaagGACTCGAGCTACGTT
-2050   GCAACGGAACAATTCCCATGTCCCTTCTCTTCTCCTGCAGGCCACACACA
-2000   GAATCTCCCGCTATCGATATCTCATCAACATGCCTGGCACTGTGTGAAAC
-1950   GCATAATATCGGGGCTGGATCTTAAAGATAACCACATGGCAACCAAAAGC
-1900   AAGCCGgtcagcattcttctagcaacatggtcgtatcatcgggtaattca
-1850   agcgtcatacctgtgactggttggtgggtgcttatttatttcttggaagT
-1800   AATTTCGTCGCCCGGCATCCCAGTCGATCACTCGCTAGTCTTGCAACAGT
-1750   TTTCTGGACGAATTCCAGGGCTGGTTTAGCGGGCCGTGAAGCTAATGCAG
-1700   CTAATCCGTTTAACCAATTGTTCCCCGAACTGGCGGTGTTGACGCACTGG
-1650   ATGCCACTGGAAGGTGGCTTGATGGCGAACTTTAACCGTCTGCCATCGAA
-1600   TTATTAACACACAAGgttcggtaattaccatactacataccatgggagca
-1550   ttagttaacactgtttcgcatgattctggtcagCAACCACTTCCTTCACA
-1500   GGGATTTGAAAGATCGAGTCCGAATGTCGGGATGTCGGCGTTCTATAGAG
-1450   AAGATACAGCAGTCGGCGTAGAGAATCATGAGCGAGACAAGAACGTATGC
-1400   GTGGAGGAATGGATATAGGAAAACCGAGATCGATCCTGCAGCAGCACCAC
-1350   AGCCGTAGGTAATAATATGCTCTGATATTGCTCTCTCCTTAGTCTCCGGT
-1300   AGGGGAACATGTCAATTTTCCGTCAGCGGGCCAGCTTCAAGAGTCTAGCT
-1250   GCAACTAAAACATAGCGGAGTCCACTACAGGATTCCTGCGCACTCCATCC
-1200   AACTGGCTTTGACGCTATTGAGGAAAAATAAGTAAATCACTAACAACGAC
-1150   CAGCTCACGCCTTTGCCGGCTGTCATGCTGTACTCATCTTTCCAACAGCA
-1100   GCAGCAGCAGAAGCAACAACAACAACAACACTGTTCCAATTCACTCCTTT
-1050   GTATGCCTTGTTACCTGACTGGGTGTCTGCTCCTCGTTCGCAGGACTCAG
-1000   CCAGATAAGCCAGCCATATGTTTTCCGTTCTCTAAAAAGTTGCTTCAGAT
 -950   GCCTCCTTGACCTCACGCAAACCTCCCTAGCAACCGCATATCCCAATGAT
 -900   TCAGCGGAAAAATAAACTCGTTGATACAAACCGTTAACCGGTCCTAATCG
 -850   ACAGTAGAACTTACATACCTAAATCCAAAAACTCCTCTCACTAATATTTT
 -800   GAACACACCAAAGGCTTCAAACCTTTCTCTCTTCACAACGAGCTCTGATT
 -750   TCCGGTGCCCACTTCGCGCAGAAATACAACAGCACAGAGCAGATTCTAGC
 -700   CCAAACTCTGAGGCATTTAAGAGTTCTCCTTATCTCGGGGTGCTCCGAGC
 -650   CATTTCACCCTCTCGTGTGGTGTTTTTTTATTTTATTTTATATTTTGTTT
 -600   AGAATTTTTTGAGAGTGTCTGAAGCTGTGGGAGGAAGCCTGTTACTGTCG
 -550   CACCGACGGCACAACCGCCAACATAAGAAGCCGTAGCTCTTGTGCCACGC
 -500   TCGGTTTCTGCACCATTGTCGTCCCCGATATCTTCACGCAACCTTATTTG
 -450   CTGTTCCGGTCCGgtatgtggagccatttgtcgacttccccgcttaacta
 -400   tgaggcaaaagttcagaatgatggtctcatctccatgctcatgcgctggt
 -350   tcatgtttaacttgcatgctgattgagttctgatagGAATTGATTGTGCA
 -300   TTTTAGCAGATTCGATTAATTGgtatgtatgctctatgcaagactatatt
 -250   caagttgtcaaatgtttttaacccttcccctttacgtctcatcccctata
 -200   attttgggttctacggcaaccatggaggtctacaatctcatagcgcgggg
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-150   catggtacaaatttgatatcacttcaaaaccacttttctctcaagccatt
-100   ctctgggtgatctcggattaagcctgattcttattcattttatcatctca
 -50   actaatgattcattctcacgacaaaaagGTTCCAATTTCATTTCTTAATC

   1   ATGGGTTGCGCAAGTTCTCAACCAGTGGATCCGACGGATAAGATTGCAGC
         M  G  C  A  S  S  Q  P  V  D  P  T  D  K  I  A
  51   TAGACACACAGCTGCGATCGACAAGATGATCCGTATGGACAAGAAGGTTC
       A  R  H  T  A  A  I  D  K  M  I  R  M  D  K  K  V
 101   AGGATCGAACGGTGAAGATTCTTTTGCTTGgtacgtcactaaatctaaat
        Q  D  R  T  V  K  I  L  L  L
 151   ctgcaactcgggtaatgatcatcactggaatttacttgttttaatattta

 201   ttcttagGTGCTGGAGAGTCTGGAAAATCAACAATAATTAAACAAATGCG
                G  A  G  E  S  G  K  S  T  I  I  K  Q  M
 251   AATTATCCATGCGGGTGGCTTCCCCGAAGACGAACGATACCAAAACCGCG
       R  I  I  H  A  G  G  F  P  E  D  E  R  Y  Q  N  R
 301   CGGTCATCTACTCCAACATGGTTGTTGCATTCAAGATTCTCCTCGATATT
        A  V  I  Y  S  N  M  V  V  A  F  K  I  L  L  D  I
 351   ATGCAGACGGAAAATATTGAATTTGAGTTTGAAAAGACAAAGgtacgaaa
         M  Q  T  E  N  I  E  F  E  F  E  K  T  K
 401   tttcgaccttgattcctgctgctaaataccgacgactctggaggttttgc

 451   ttatataaatgccgtaaagCCACTAGCCAAGCTATTAGACCAGACAGAGC
                            P  L  A  K  L  L  D  Q  T  E
 501   CGGACGTGGACACAAACGAAGCTTTTAGTGATCTCGCCGTCAAAGATGCA
        P  D  V  D  T  N  E  A  F  S  D  L  A  V  K  D  A
 551   ATGAGTGGAATGTGGCTAGATAGGGGCGTACAGAAAGCTGTTTTACGAGG
         M  S  G  M  W  L  D  R  G  V  Q  K  A  V  L  R
 601   ACACGAATTTGCACTTCATGATAATCTTCAGTAgtacggatttccttttt
       G  H  E  F  A  L  H  D  N  L  Q
 651   tttttttttttgatatcatggatatttcgggagatatatcaattgacata

 701   caacacagCTATTTCAAATCTCTCAACCGCGTCTTTACCCCTGGGTGGTT
                Y  Y  F  K  S  L  N  R  V  F  T  P  G  W
 751   ACCCAACAATCAAGACATGCTTCATTCCCGACTTCGTACCACAGGCATCA
       L  P  N  N  Q  D  M  L  H  S  R  L  R  T  T  G  I
 801   CAGAAACCTTATTTGAATTGGGACAAATAAATTTCCGAATGATGGACGTT
        T  E  T  L  F  E  L  G  Q  I  N  F  R  M  M  D  V
 851   GGCGGGCAACGATCGGAGCGTAAGAAGTGGATTCATTGTTTCGAGGGGGT
         G  G  Q  R  S  E  R  K  K  W  I  H  C  F  E  G
 901   GCAGTGTCTTCTATTTATGGTTGCTCTGTCAGGTTATGATCAATGCCTTG
       V  Q  C  L  L  F  M  V  A  L  S  G  Y  D  Q  C  L
 951   TTGAGGATCAAAATGCAgtaagttcgaaattcccccccttttaacaaaat
        V  E  D  Q  N  A
1001   gtataaataaatggaaaaccttagcatggcttacatataattatgttgtt

1051   gacagAATCAAATGCATGAAGCCATGATGCTATTCGAATCTCTGGTAAAC
              N  Q  M  H  E  A  M  M  L  F  E  S  L  V  N
1101   GGGGAGTGGTTCAAACGGAAACCGGTGATTCTATTTCTCAATAAAATCGA
         G  E  W  F  K  R  K  P  V  I  L  F  L  N  K  I
1151   CCTCTTCAAGGATAAGGTTTCTATATCCCCAGTGTCTAAATTCTTCCCGG
       D  L  F  K  D  K  V  S  I  S  P  V  S  K  F  F  P
1201   ATTACCATGGCGCAGACGGCGATTATGATGCGGCTGCTAAGTATTTCTCT
        D  Y  H  G  A  D  G  D  Y  D  A  A  A  K  Y  F  S
1251   GACCGATTTCGCGGCATCACCCGCGTCCCTGAACGGGAGATCTACATCCA
         D  R  F  R  G  I  T  R  V  P  E  R  E  I  Y  I
1301   CTACACAAATGCTACTGATACCACCCTATTAAAGGCCACGATGGATTCAG
       H  Y  T  N  A  T  D  T  T  L  L  K  A  T  M  D  S
1351   TGCAGGACATGATTATCCAGAAGAACCTCAACAACCTGATTCTGTAGGTG
        V  Q  D  M  I  I  Q  K  N  L  N  N  L  I  L  *
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1401 CCAACAGTGCTAGAAATGAAACTGCAACGGAGCTCCTAGTAGGAGACAAT

1451 TTATTTTGCCGTGATACCGAATAGAGTCTGCTATTATCACATTAAGTCTG

Polyadenylation site

1501 ACACCCCCCTCCACCGCTCCCGGGATTTCTCCACGCACCCTGTCTCATCT

1551 CCTTTTCGTTTCCTTTTGATGGCTAAGATTTTTTTTCTTTTTTCTTTTTT

1601 CTCTCTTTTTTTTTTCTATTCTTTTTTTTAATGGCC

Notes:

Nucleotide positions are written in red numerals, amino acids in blue.

Introns are denoted by lowercase letters with red color.

Polyadenylation site is underlined.

Figure 3.7-3 The nucleotide sequence and the deduced amino acid sequence of *PbGPA3*

```
-700   TCTCTCTCTCTCTCTCTCTCTGTGGCGCTGAACGCTTCCCAATATCCT

-650   CTACCTTCTCCATCCTCGCCTTCCATTCAGCCTCTTCAACCCACCCTACC

-600   ATAACCCTGTCCAACTCCACGAACTTCTCCCTCCTGCATTCATCGAAGAA

-550   TTATCCCGCATCGGGCCGCTTACTTATCAAATCGACAATACCTTATTGCA

-500   ACCTACTTATTCCCAGGCCAAACTATGTGAGCCACCGTATAACTCCCCAG

-450   GCCACTCACTGCTTTCTTTGCCGATCTCGGCTTTGTGGTTCATTGTTCCA

-400   GATATCTCACGATCAACCGTCTATCCATCTGACAATCTAGCCTCCTTGGC

-350   TTCTCCATATTCCCAGAATCTATCAGAATCTACAAACTACCGTATAATAC

-300   CTTCTTCGCCTCAGTCGGTCTCCGTTCCGTGGCTTCAGGCCCAGGACGGC

-250   TGGCTGCCTCGATCTCAAATAATACACACAGTGTCCCTCCCGGTCCCATC

-200   TCGATTTGATCGCGCCTTTCCATCTATCTGCCCTTGCTACCCGAGATTTT

-150   GTTTCGAGGTTATAATCAGGCGTGTAGTGCATTCCTAATATCGTCTCCAT

-100   ACATCCATAGAGGCGCCTTACATCTCTCTCCTGTTGTGTGGTTGGTGTAT

 -50   TAAGGTCATATTGTTCGCCCTGTTAGTAGAAGCATTCGATATCAGATAGG

   1   ATGGGTGGGTGTTGCAGTTCTGCTTCTGGGGAGAATGCAGAGCAGAGGAA
         M  G  G  C  C  S  S  A  S  G  E  N  A  E  Q  R
  51   GAGAAGCCAGATGATTGACAAGGGGCTCGAGGATGATTCAAAACGATTAC
       K  R  S  Q  M  I  D  K  G  L  E  D  D  S  K  R  L
 101   GGAGAGAATGCAAGATCCTACTGCTTGgtctgtcattaccgtccttttct
        R  R  E  C  K  I  L  L  L
 151   tcttcacccccagtcacgggtgcattggccttgccaacttaccaactttg

 201   cactctgttgagtgtctgtgtgtctgttagatgtgtaaacagtggatatg

 251   ttctgacggtcattacagGTTCGGGTGAGAGCGGAAAATCGACGATCGTA
                           G  S  G  E  S  G  K  S  T  I  V
 301   AAGCAGATGAAAATTATCCATCAGAACGGATATTCTGTCGAAGAACTTAC
         K  Q  M  K  I  I  H  Q  N  G  Y  S  V  E  E  L
 351   CTTGTGCCGTTCAACAGTCTACAAAAATCTCTTGGATTGCGCCAAGGACC
       T  L  C  R  S  T  V  Y  K  N  L  L  D  C  A  K  D
 401   TGATAGGAGCTTTACATCATTTCGACTTACAGCCATCCAGTCCCAAAGTT
        L  I  G  A  L  H  H  F  D  L  Q  P  S  S  P  K  V
 451   CAAGATTATATAGAGTATCTTAGTTCATACCAGATTGACGCGGACCCGAA
         Q  D  Y  I  E  Y  L  S  S  Y  Q  I  D  A  D  P
 501   TACGCCAATAGATCCGAAAGTGGGTGATGCCGTGACATATCTCTGGAATG
       N  T  P  I  D  P  K  V  G  D  A  V  T  Y  L  W  N
 551   ATCCTATCATGCCATCCGTGCTCGAACATCAGAACGAGTTCTACCTCATG
        D  P  I  M  P  S  V  L  E  H  Q  N  E  F  Y  L  M
 601   GATTCAGCACCTTAgtatgccctcccccttttcactggagtctttgcttt
         D  S  A  P
 651   gcgatgttctaatcattctcagCTTCTTCGAGGAAGCCAAACGTATTACC
                              Y  F  F  E  E  A  K  R  I  T
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701   GCACCCGACTACATACCTAATGAAGCCGATGTGTTGCGCGCTAGAACAAA
        A  P  D  Y  I  P  N  E  A  D  V  L  R  A  R  T
751   GACAACTGGTATATATGAAACGAGATTCACAATGGGCCAATTGAGTATTC
      K  T  T  G  I  Y  E  T  R  F  T  M  G  Q  L  S  I
801   Agtgcgtttaaatatatcacccctactttgccggaacatgaccctaatat

851   gtatatcttttagCATGTTCGATGTTGGCGGGCAGCGGAGTGAACGGAAA
                     H  M  F  D  V  G  G  Q  R  S  E  R  K
901   AAGTGGATTCACTGCTTTGAAAACGTAACCTCCATAATATTCTGCGTCGC
        K  W  I  H  C  F  E  N  V  T  S  I  I  F  C  V
951   ATTGAGCGAGTATGATCAAATGTTACTAGAAGAAAGTAATCAAgtatgcg
      A  L  S  E  Y  D  Q  M  L  L  E  E  S  N  Q
1001  atgaccctcatttcctcgcgttacctctattgcgttgtttactgggtcta

1051  accttgacgaaagAACCGCATGATGGAGAGTTTAGTGCTGTTTGATTCCG
                        N  R  M  M  E  S  L  V  L  F  D  S
1101  TTGTCAATTCCCGATGGTTTATGCGGACGAGTATAATTCTCTTCTTAAAT
       V  V  N  S  R  W  F  M  R  T  S  I  I  L  F  L  N
1151  AAGGTCGACCTCTTCCGGCAAAAACTCGACCGGTCCCCATTGAATAAATA
        K  V  D  L  F  R  Q  K  L  D  R  S  P  L  N  K
1201  CTTTCCCGATTACTCTGGAGGAAACGACGTCAACCGTGCCGCCAAGTACT
      Y  F  P  D  Y  S  G  G  N  D  V  N  R  A  A  K  Y
1251  TGCTCTGGAGATTTAACCAAGTTAACCGGGCGCATTTGAATCTTTATCCT
       L  L  W  R  F  N  Q  V  N  R  A  H  L  N  L  Y  P
1301  CAgtatgtataggaaaagcacaccttaattacttcttcctttgttttcaa

1351  aaagcccttgtcctgtacctaacctccccgctgcggcagCCTTACTCAAG
                                             H  L  T  Q
1401  CCACCGACACGTCGAATATTCGCCTCGTCTTCGCAGCCGTGAAGGAAACC
       A  T  D  T  S  N  I  R  L  V  F  A  A  V  K  E  T
1451  ATTTTACAGAACGCCCTGAAAGATTCTGGTATTTTGTGAAAGGGAGAAAA
        I  L  Q  N  A  L  K  D  S  G  I  L  *
1501  CGAAGAAAACAAAGCAAAAAGGAAAACAAATACACCACGAATTGTCGGAA

1551  TTTTTTGTTCCACCTTGATATTTTTTTTTTGGAAGGCTGCGGGGGTCGTC

1601  TTAGCCATCGAGAGCGAATATTCTATACCCCGCCGTTCCGNACCGCATTT

1651  CCGTTTCCGCCGCAAATTATTACTTACTTGCAAATTCTTCACCGGTTTGA

1701  CTTACGATGACGACACCATTACCCACTACCCACCACCGTCTACAAAAAAA

1751  ACGCCATAATAATGTCTTTACCCCTCTACATGCTTTTCAAACTTGTGCTT

1801  CATCAAGTGCCCGGTGCTTCTCTCTGTATACAGCCAGCNACGCTTTTAAA

1851  CGCCAACTTTTGGATACCAGCCCGGGCCGGAA
```

Notes:

Nucleotide positions are written in red numerals, amino acids in blue.

Introns are denoted by lowercase letters with red colour.

Figure 3.7-4 Comparative alignment of PbGpa1, 2 and 3. Protein sequences of PbGpa1, 2 and 3 are aligned by using VectorNTI (Informax). Positions where introns are spliced are indicated with black vertical bars. PbGpa1, 2 and 3 have intorn-splicing sites at the same positions as indicated by the yellow triangle.

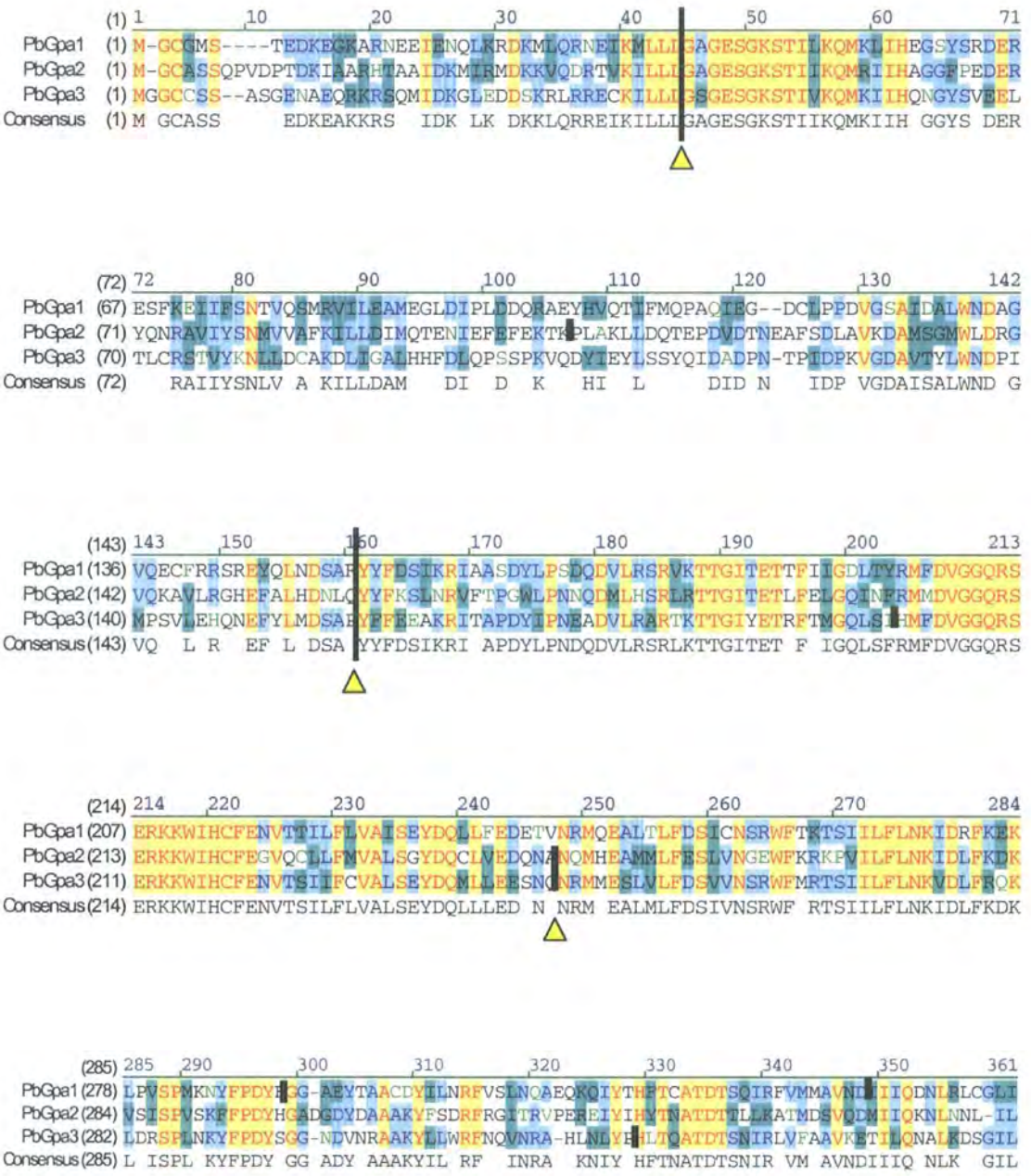
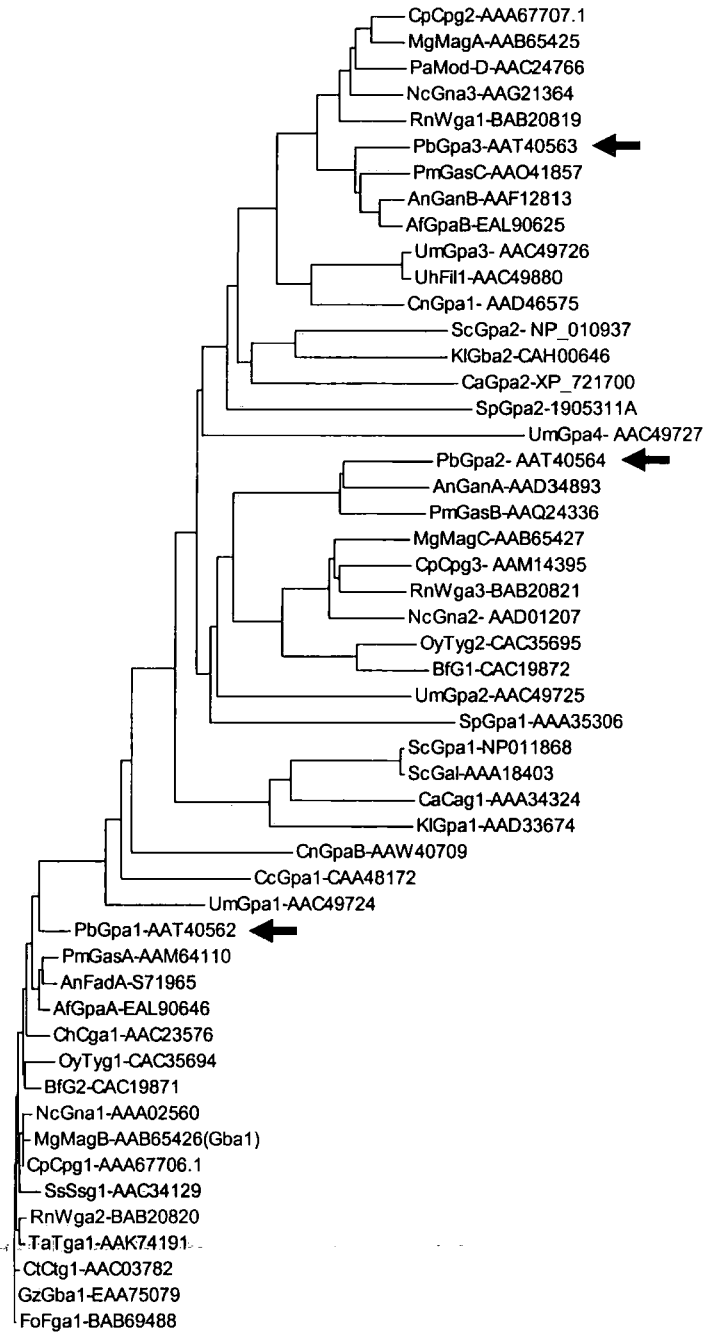


Figure 3.7-5. The phylogenetic relationship between fungal Gα-proteins. A neighbour-joining bootstrap tree is derived from the amino acid sequences of all the fungal Ga-proteins using the VectorNTI 6.0 align program. The GenBank accession numbers of the Gα-proteins are indicated following the abbreviated name of the protein. The abbreviations are as follows: Pb, *P. brasiliensis*; Af, *Aspergillus fumigatus;* An, *A. nidulans*; Bf, *Botryotinia fuckeliana;* Ca, *Candida albicans;* Cc, *Coprinellus congregatus;* Ch, *Cochliobolus heterostrophus;* Cn, *Cryptococcus neoformans*; Cp, *Cryphonectria parasitica*; Ct. *Colletotrichum trifolii;* Fo, *Fusarium oxysporum*; Gz, *Gilbberella zeae;* Kl, *Kluyveromyces lactis;* Mg, *Magnaporthe grisea*; Nc, *Neurospora crassa*; Oy, *Oculimacula yallundae;* Pa, *Podospora anserine;* Pm, *Penicillium marneffei;* Rn, *Rosellinia necatrix;* Sc, *S. cerevisiae*; Sp, *S. pombe*; Ss, *Sporothrix schenckii;* Ta, *Trichoderma atroviride*; Uh, *Ustilago hordei;* and Um, *Ustilago maydis*.



3.8 Cloning of a G Protein β Subunit Gene *PbGPB1* from *Pb01*

A G protein β subunit gene *PbGPB1* has been cloned using a degenerate PCR approach. Two degenerate primers, PbGPB-F1 and PbGPB-R1, corresponding to conserved amino acid residues AKIYAMHW and WVMTCAYA, were used to amplify a 153 bp fragment from *Pb01* genomic DNA. The fragment was sequenced and specific primers were designed. Fragments upstream and downstream of the gene were amplified from both our genomic walker library and pDNR-*Pb01*cDNA library. The cloning of *PbGPB1* was later finished by Dr G. Chen in our lab.

Figure 3.8-1 illustrates the genomic DNA, cDNA and the deduced amino acid sequence of *PbGPB1*. The coding sequence of *PbGPB1* is 1,059 bp long and is interrupted by 4 introns. The gene encodes a protein of 353 amino acid residues with a molecular weight of 39 kDa and predicted pI of 6.8.

G protein β subunits are highly conserved from humans to fungi (Figure 3.8-2 and Figure 3.8-3). When analysed with EMBL's SMART, G protein β subunits contain a conserved coiled-coil domain (SpGit5 is an exception that lacks this domain) and 7 conserved WD40 domains as illustrated in Figure 3.8-2. Phylogenetic analysis indicates that PbGpb1 is closely related to AnSfaD (Figure 3.8-3). Interestingly, the distance between PbGpb1 and the G β subunits of *S. cerevisiae* (Ste4) and *S. pombe* (Git5) is further than the distance between PbGpb1 and the G β subunits of humans (e.g. Gnb1, Bnb2, Gnb4 and Gnb3). The percentage of identity to PbGpb1 is as follows:

Protein: organism	**% identity to PbGpb1**
AnSfaD: *A. nidulans*	93%
CpCpgb1: *Cryphonectria parasitica*	83%
MgMgb1: *Magnaporthe grisea*	81%
NcGnb1: *Neurospora crassa*	81%
UmBpp1: *Ustilago maydis*	72%
CnGpb1: *Cryptococcus neoformans*	70%
HumanGnb1: *Homo sapiens*	66%
HumanBnb1: *Homo sapiens*	64%
HumanGnb4: *Homo sapiens*	63%
HumanGnb3: *Homo sapiens*	62%
Ste4: *S. cerevisiae*	35%
Git5: *S. pombe*	37%

Figure 3.8-1. The nucleotide sequence and deduced amino acid sequence of *PbGPB1*. Nucleotide positions are written in red numerals, amino acids in blue. Introns are denoted by lowercase letters with red colour.

```
-1800   GCACTTTGACGTTGCAGGATGTTCCAAAATAAAGAATTAACTATCCATAG
-1750   GGAGGCAAGTTGGGTAGGTGCCTGCGTTGGAAAAGAAATCTCATGTCGAC
-1600   TCATTGTATTTGCTTATTGACCCATATATCGATAACTATTGCACCTCCGA
-1550   AAAAGCCGAGGAAATCGGTAACTTGTCAACATGTCAAAAACTATTTTGGT
-1500   GGAGCATAGACACGGGATAGTTACTAATAGAAATGTTGTTTTATGAATAG
-1450   GCAAGGCACTGATGCTTCGTCATTGAGAACTGACAATTTCCCGAAATCCC
-1400   AGCAAACTCCAATGACCCAGCTCCATTGGTTTTTTTTTTTTTTTTTTTT
-1350   GGTGGCCCCTTCTGGAAACCGTCTCCAGAATGCTGGTGTCGAGGAAGTCC
-1300   GTCCCCAAATGATTTGACAATTGCGTCTGGCTAGGTCCTGCCAAGGCGCC
-1250   TGAAGAAGTCTTCCATGGAAGTTTTTTGCTTTCCACAAACACCCTGGCGA
-1200   CTCTTAGGCAAGCTGGTGTTTCTGACGTGCCAATAGGACAGTAAGTAATA
-1150   ATAAAGGGTGTTCCAATCATCTACAACCCTATGTTTGCTGACACCGAGCA
-1100   CTGAGCACTGAGCAGTCGAAGGAACGGGAGGAAAGAAACTCAAAAATTGT
-1050   AACAAAAAGGAGAATAGATAACCGTGTCAGTCCTCAAATAAGACGGGTGG
-1000   TTGGAATATTTCCTGGTGTAGCCTTCTGTATCAATCGCTTTTCAGCCACA
 -950   GCAGGGCAGATGCATGAACGCTGTTAATATTCAGACATAGGAAAAAAACT
 -900   TTGTATTTAACTTATCGGTGTCATTCCCTTGGTTTGTCAGCAGCGGATAT
 -850   TTGTGGAGTAGAATACCGGGGATTAAATACAGCAGATATACCGACAGATT
 -800   ATGGGCACAATACAACCCAAGTACATCCACGGGATATAGTGTATGGATAG
 -750   ATACAGTTCTGTAGGCAAACGCACTCTGTACCGTGAGTGTAATATTACTT
 -700   ACCCTAGGTACCGCCCAAACCAGTTCCAAACCAGCACAACCACTGTCTTC
 -650   ATCAGCCTCAACCCTTGAACCTCATCCTCATCTTCATCTGCTCCTCTCAC
 -600   TCACTCACCCTAATTTACCCAAACGCCTCACACATCCCCAGACTGGAATT
 -550   TCTCCTATCCCTCTCCTCTCTTCAATCTTAGTTGCCCCCTCGATTCTGCC
 -500   TGCTGCTGGTCATCGTGGATTGCTTGCTCCCCAGTCCTCTCCTCTCACCC
 -450   TGCTGCCAGCCATCCCTGAGCATATCCAGTGGTTGGTGTGGCCATTTGCC
 -400   CTGTCTGTGCTGTGGTAGAGGGGACGACTCTTCTGTTTACTATATTCAAA
 -350   GATCTGTCACCTTCGCATCTGGTGCTTCCACCACTGCAACTGCTGGAGTC
 -300   CCAAGTTCTCCACTCTATCTCATTAACCATTATTCCAGCCAACCACCCTC
 -250   TCTGCCTCCTGTCCCAGGGCCCCCAATAATAGTGCTATTGGTGCAATCCC
 -200   AGCTAGGTCGCTTTCTAGTTCCCACATCATTTCTCCCAACCACAACCACA
 -150   ACGGTTACCACAACCCTCCACTGCCTCCTCCCCCGATCGACCATTCGCCA
 -100   AATTTTGCTCTCCGTTAAACTACCACCCTTCTTCCTATCCATCCTCTTCC
  -50   CATCCAGTTGACCACCCTCTCTCTATTCTTTGTGGTCTCGTTAATCTACC

    1   ATGGCGGCCGATTTGAGCGGCGAGCAAATGCAGGCGAAGATCACCGCTGC
    1     M  A  A  D  L  S  G  E  Q  M  Q  A  K  I  T  A
   51   CCGTCGTGAGGCAGAGGGTCTAAAGGATCGCATCAAGCGCAGGAAAGATG
   17   A  R  R  E  A  E  G  L  K  D  R  I  K  R  R  K  D
  101   AGCTTGCGGATACCTCTCgtaaggactgctgtctcttttactcttcaccc
   34    E  L  A  D  T  S
  151   catctccacatatgttatcacccaccatggcatctatgaactcctctgaa
  201   tctctctttacacaactctcgtccattgaacccagccaagtcataagagt
  251   tttctcactcgttggggctttctcgcacatgtccaccagtcattggctct
  301   atccccatcccgaggttaaacccaggccattggttaattaatactgacat
  351   aacataccttcctcatagTTCGCCAAGTCGCACAGAACAATACGGACCCC
   40                     L  R  Q  V  A  Q  N  N  T  D  P
  401   CTTCCACGAATTGGAATGAGACCGCGACGGAACTTAAAGGGCCACTTGGC
   51     L  P  R  I  G  M  R  P  R  R  N  L  K  G  H  L
  451   CAAGATCTATGCGATGCACTGGTCGACAGACCGCCGCCATCTGGTTTCTG
   67   A  K  I  Y  A  M  H  W  S  T  D  R  R  H  L  V  S
  501   CCTCTCAAGACGGGAAACTAATCATCTGGGATGCATACACAACAAACAAA
   84    A  S  Q  D  G  K  L  I  I  W  D  A  Y  T  T  N  K
  551   GTGCACGCCATCCCCCTAAGATCGTCATGGGTGATGACTTGCGCCTATGC
  101     V  H  A  I  P  L  R  S  S  W  V  M  T  C  A  Y
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```
601   CCCGAGCGGTAACTATGTCGCCTGTGGTGGTCTAGACAACATCTGTTCCA
117   A  P  S  G  N  Y  V  A  C  G  G  L  D  N  I  C  S
651   TCTATAATCTTTCCTCTCGAGAAGGTCCAACCCGTGTCGCACGAGAATTG
134    I  Y  N  L  S  S  R  E  G  P  T  R  V  A  R  E  L
701   TCCGGCCACACCGGTTACCTGTCATGTTGTCGTTTCGTAAATGACCGTCG
151      S  G  H  T  G  Y  L  S  C  C  R  F  V  N  D  R
751   AATCATCACTTCTTCCGGCGACATGACCTGTATGCTATGGGATATCGAAT
167   R  I  I  T  S  S  G  D  M  T  C  M  L  W  D  I  E
801   CGGGGTCGAAAGTCACTGAATTCGCCGATCATCTGGGTGATGTTATGAGC
184    S  G  S  K  V  T  E  F  A  D  H  L  G  D  V  M  S
851   ATCAGCATAAACCCGACAAACAACAACGTTTTCGTCTCCGGCGCATGCGA
201      I  S  I  N  P  T  N  N  N  V  F  V  S  G  A  C
901   TGCCTTTGCTAAACTCTGGGATATCCGCGCTGGGAAGTCGGTACAAACAT
217   D  A  F  A  K  L  W  D  I  R  A  G  K  S  V  Q  T
951   TCTCCGGTCATGAATCGGATATCAATGCCATCCAGTTCTTCCCTGATGGC
234    F  S  G  H  E  S  D  I  N  A  I  Q  F  F  P  D  G
1001  AATGCTTTTGGTACCGGATCGGACGACACCACCTGCCGTCTATTCGATAT
251      N  A  F  G  T  G  S  D  D  T  T  C  R  L  F  D
1051  CCGCGCGGATAGAGAACTTAATACATACCAGgtaatattattaattcaaa
267   I  R  A  D  R  E  L  N  T  Y  Q
1101  cgcctcatgacttaaattgtcaacataaaagttcgttggataactgacat
1151  gatcttttatattatacgcagAGTGATCAAGTACTTTGCGGTATTACTTC
278                           S  D  Q  V  L  C  G  I  T
1201  CGTTGCGTTCTCAGTCTCTGGTCGACTTTTGTTTGCTGGCTACGATGATT
287   S  V  A  F  S  V  S  G  R  L  L  F  A  G  Y  D  D
1251  ATGAGTGCAAGgtaagtcaacctatttatttgctatccctttgtgcgaa
304    Y  E  C  K
1301  ccaaactaattaatacgtaatcttttcaagGTCTGGGACGTTCTAAGAGG
308                                    V  W  D  V  L  R
1351  AGAGAAGGTCGGTTCACTGAGTGGCCATGAAAATCGTGTCAGCTGCCTGG
314   G  E  K  V  G  S  L  S  G  H  E  N  R  V  S  C  L
1401  GCGTCAGCAACGATGGAATCAGCTTGTGCACAGGATCTTGGGATTCTCTG
331    G  V  S  N  D  G  I  S  L  C  T  G  S  W  D  S  L
1451  gtgagcgttcaatgattagttcaattctacagtgaagttccccttgctga
1501  tatatggtttttttccaatcttcagCTCAAGGTCTGGGCATGGTAGACTT
348                              L  K  V  W  A  W  *
1551  GGTTTTGGCCAAATTATCGATCGTTGATGGTGGAACTGAACGAACACCCC
1601  ACACCACCCGCCTTCAATTTTGCAGTTCTCGTACCACACACCCTCCCTCC
1651  CTCCCATCGACAAGGAAGTTTTTTCGCCTACAACATTCATATTCCGTATA
1701  TCCGTTTCTCTCCCACACAAACACCATGATATCCTCGAGGAACTGTGCCA
1751  TCTACAGGACAGGCATATATAATTGCAAGCATGCTTCTCAAAAATTTAAC
1801  TACAGGGGGACGGACTCGAGCTTCTCGTCTCTTTCTCACAACAGCCTTCT
1851  TACACTCGTAAGGCGACACAGAATAACATCAAACCTATGATGACATGAAG
1901  GAAACAAACCTTGTTCCTTAATCCTCGCCATCCCTGCACACCTCTCTGAC
1951  ACTATAACCCCCTTCTTTTGTCCCTTAAGTGTCTTAATTAGTGTCTTAAT
2001  TTTCGGAACTAGAAGAAGATGAAAAGAGAGGAGTCAAGATCCTACCTTTT
2051  ACTTTCTGCTCACAAAAAGCTCACGAAACGAAAAGAAAGACACTAGGAGG
2101  AATAAAAAATTGAGACTCTTGAGCTGCGAGATATCATTTGGATAATAATC
2151  TTTTTGATGGGATTTTCCTTCATTTTTTGTTCTTCACTTCAGTATATCCA
2201  AGTACCTAATTTCTGGTTGCCACTTTTCCAGCTTTTGTTGATGTTTTCTG
2251  TCTTTTTAAATGGCTCTCTGTTCTCCCCCTTACTGTTTACCTACACATAC
2301  ACCGTTTTACTTTTCTTTATGGNGCTGGTTTCCTTATTTTCCTCCCGTTA
2351  ATTTTTTATACCTCGGGCTTGGAAAGGATTGTGAGCCTTTATTATTTTCC
2401  TTATAGTCTTTTGCTCCGTTGTTTTAAACCCCCATTATCAGGCTATAAGT
2451  TTCTTCATTTTTCTTCCCTACTTTCTTTGCTGCCGGGTTTCTCTCTCTCT
2501  TTCTCTCTCAAAAATTCTTTTTGGGGATTTCTTCGTCGTTTCTTCCTTGT
2551  TTGATTCCTACATTCCGAATTGCCTAGGTGTGTATTACCTTTTTCCCCCT
2601  TTTCTTTCTTTCTCTATAGCCATTGGTCCTCATGTACTCCTGATATTTCC
2651  TCCTAGGTCCTGTTCTTAACCCCACGTTCTATTTTCCATTATGTTTTCCT
2701  TATCTCC
```

Figure 3.8-2. Comparative alignment of PbGpb1 with a G protein β subunit of human being and the Ste4 protein of *S. cerevisiae*.

The alignment was done with the software Vector NTI 6.0 (informax).

G protein β subunit is conserved among different organisms. Its characteristic is the 7 WD40 repeats which form a circular beta-propeller structure with 7 blades. The repeating units serve as a rigid scaffold for protein interactions. The specificity of the proteins is determined by the residues outside the repeat structure.

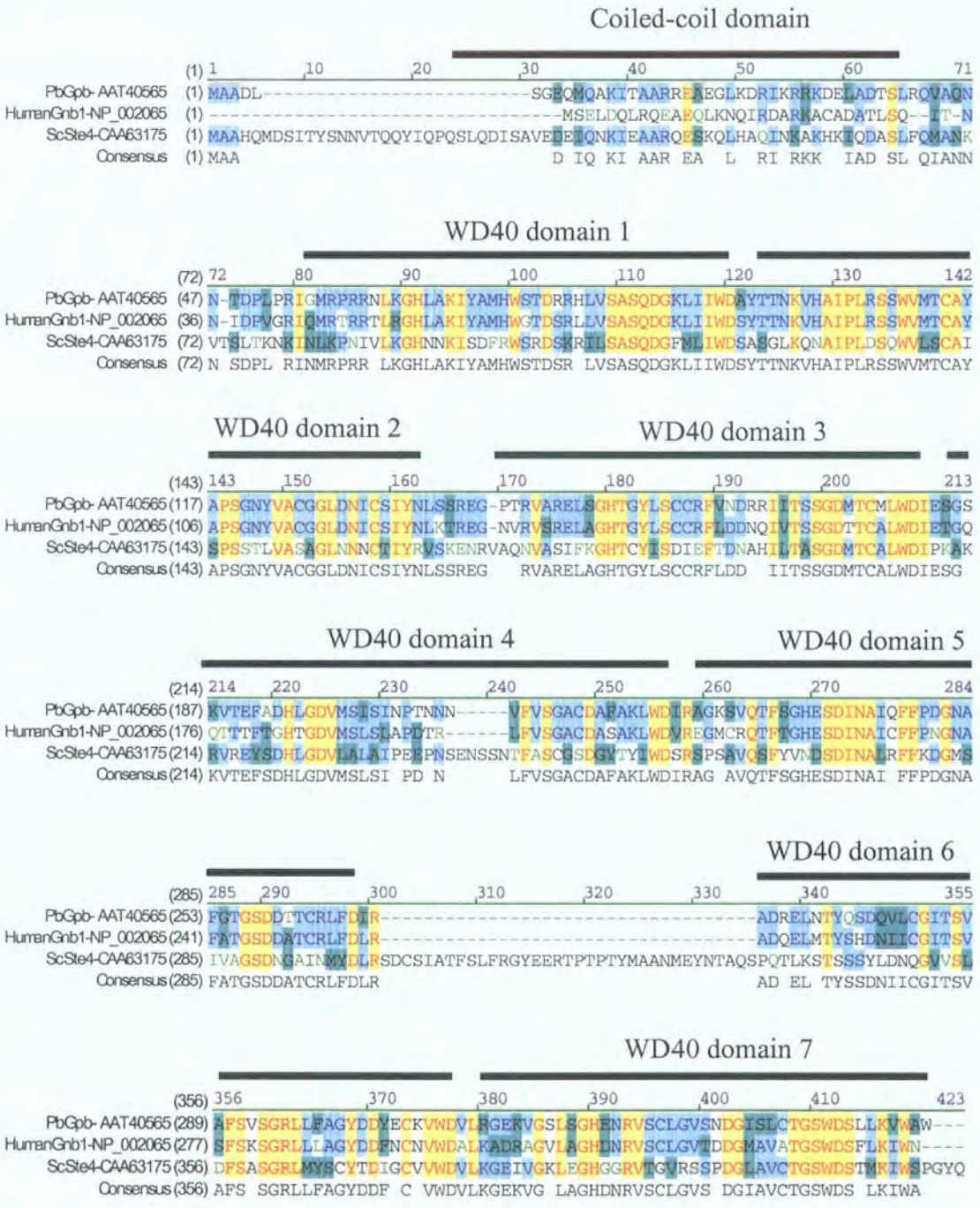
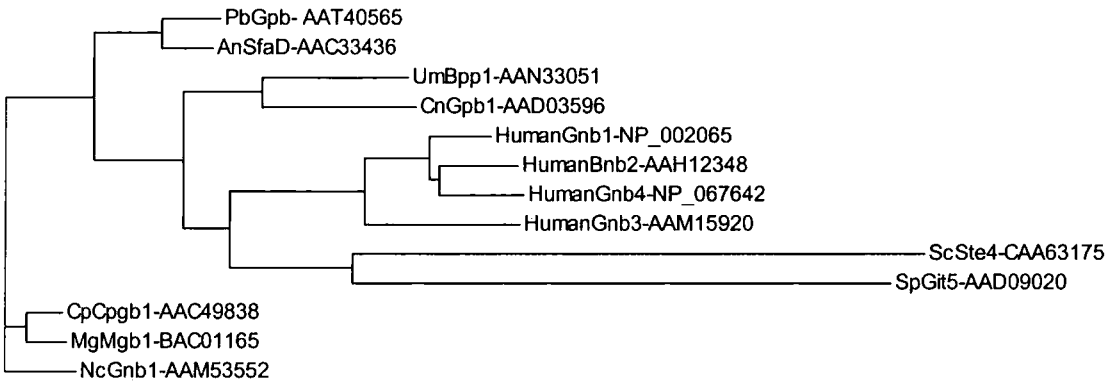


Figure 3.8-3. Phylogenetic analysis of fungal and human G protein β subunits. The tree was generated with the programme Vector NTI 6.0 (Informax). GenBank protein accession number was given after the protein name. The abbreviations are: Pb, *P. brasiliensis*; An, *A. nidulans*; Cn, *Cryptococcus neoformans*; Cp, *Cryphonectria parasitica*; Human, *Homo sapiens*; Mg, *Magnaporthe grisea*; Nc, *Neurospora crassa*; Sc, *S. cerevisiae*; Sp, *S. pombe*; and Um, *Ustilago maydis*.



3.9 Cloning a cAMP-Dependent Protein Kinase Catalytic Subunit Gene *PbTPK1* and a cAMP-Dependent Protein Kinase-Like Gene *PbTPKL1* from *Pb01*

The strategy for cloning of *PbTPK1* was PCR amplifications walking on both genomic DNA and cDNA (Figure 3.9-1). Primers were designed on the basis of the immediate sequencing results. Initially, 2 degenerate primers, PbTPK-F1 and PbTPK-R1, were designed that corresponded to conserved amino acid residues HPF(IL)IRMWG and DFGFAK(YE)V, respectively. A single fragment, namely gPbTPK-F1R1, was obtained. The fragment was sequenced and 2 specific primers, PbTPK-F40 and PbTPK-R5, were designed. Using PbTPK-F40 and the anchor primer M13R1, a downstream cDNA fragment, named cPbTPK-F40M13R1, was amplified by SSP-PCR from our pDNR-*Pb01*cDNA library. The cPbTPK-F40M13R1 cDNA fragment contains a poly(A) tail, indicative of the end of the cDNA. An upstream cDNA fragment, cPbTPK-R5M13F1, was amplified in the same way, by using PbTPK-R5 and the anchor primer M13F1. Further upstream amplification of cDNA, using SMART RACE RT-PCR, was unsuccessful. Based on the sequence of cPbTPK-R5M13F1, primers PbTPK1-R8, PbTPK1-R10, PbTPK1-F7 and PbTPK1-F8 were designed and used to amplify 2 genomic DNA fragments, namely gPbTPK1-R10AP3 and gPbTPK1-F7AP4, from our *Pb01* GenomeWalker library. A genomic fragment, gPbTPK-F6R11 connecting gPbTPK-R10AP3 and gPbTPK-F7AP4, was amplified with primer PbTPK1-F6 and PbTPK1-R11. Primers PbTPK-R13 and PbTPK-R14 were synthesized based on the sequence of gPbTPK-R10AP3 for further upstream genomic walking. A fragment, gPbTPK1-R14AP3, was amplified from our GenomeWalker library by SSP-PCR using the anchor primers AP3 and AP4.

In order to search for additional iso-forms of *PbTPK* genes, another 4 degenerate primers were synthesize; namely TPK-DF1, TPK-DF2, TPK-DR1 and TPK-DR2, corresponding to amino acid residues GTGSFGRVH, QVEHTNDE, YRDLKPEN and DVKSHPWF, respectively. Eight fragments were amplified from both genomic DNA and cDNA by different combinations of forward and reverse degenerate primers. Sequencing results indicated that all these fragments have the same sequence as *PbTPK1*.

In order to check if there is a *TPK*-like gene in *P. brasiliensis*, 5 degenerate primers were designed based on the sequences of *TPK*-like proteins from *Aspergillus nidulans* and *Candida albicans*. These were 3 forward primers TPKL-DF1, TPKL-DF2 and TPKL-DF3, corresponding to amino acid sequence PYVVC(VS)FE, SE(IL)D(VI)SVYD, (ED)AFLGHVR respectively; and 2 reverse primers TPKL-DR1 and TPK-DR1, corresponding to amino acid sequence TKMVDFWS and YRDLKPEN. Single band amplifications were obtained from both genomic DNA and cDNA by degenerate PCR with different combinations of forward and reverse primers. Sequencing of these fragments indicated that they have the same DNA sequence. The deduced amino acid sequence is highly conserved with that of Tpk-like proteins from *Aspergillus nidulans* (AnSchA, GenBank accession number AAK71879; Fillinger et al, 2002) and *Candida albicans* (CaSch9, GenBank accession number EAL02261). The gene was thus named *PbTPKL1*, which is illustrated in Figure 3.9-2.

The nucleotide and the deduced amino acid sequence of *PbTPK1* are illustrated in Figure 3.9-3. The 5' end of the cDNA was not cloned (Figure 3.9-1). Its sequence

was deduced from the sequence of genomic DNA. The start codon ATG was determined by finding a read-through in the genomic DNA ahead of the cloned cDNA. Interestingly, the deduced start codon ATG does not meet the requirement of a Kozak sequence "ANNATGG" (Kozak, 1987; and Kozak, 1991). However, only 1 Kozak sequence is found in the upstream genomic DNA sequence (-2306—-2300 in Figure 3.9-3). If this ATG were used as the start of translation, the cDNA of *PbTPK1* would be too long to be possible. PCR or a RT-PCR needs to be done to confirm the start of translation. The coding sequence of *PbTPK1*, which is interrupted by 3 introns, is 1,749 bp in length and encodes a protein of 583 amino acids with a molecular weight of 65 kDa and predicted pI of 8.4.

A phylogenetic analysis of fungal cAMP-dependent protein kinases and cAMP-dependent protein kinase-like proteins indicated that PbTpk1 is closely related to AnPkaA, NcPkaA, and MgCpkA; while the 3 cAMP-dependent protein kinases from *S. cerevisiea* and 2 from *C. albicans* constitute another related group (Figure 3.9-4). Figure 3.9-4 also shows that cAMP-dependent protein kinase-like proteins from *P. brasiliensis*, *A. nidulans*, *C. albicans*, *S. cerevisiae* and *S. pombe* form another group distantly related to fungal cAMP-dependent protein kinases. Interestingly, the second PKA catalytic subunits of *A. nidulans*, *N. crassa* and *M. grisea* form another group which locates between their first PKA catalytic subunit group and the kinase-like protein group (Figure 3.9-4). Proteins related to PbTpk1 are displayed as follows:

Protein: organism	**% identity to PbTpk1**
AnPkaA: *A. nidulans*	54%
NcPkaA: *N. crassa*	54%
MgCpkA: *M. grisea*	52%
ScTpk2: *S. cerevisiae*	43%
ScTpk3: *S. cerevisiae*	39%
ScTpk1: *S. cerevisiae*	39%
CaTpk3: *C. albicans*	40%
CaTpk2: *C. albicans*	42%
CnPka1: *C. neoformans*	42%
SpPka1: *S. pombe*	38%
UmUka1: *U. maydis*	28%
AnPkaB: *A. nidulans*	29%
NcPkaB: *N. crassa*	26%
MgPkaB: *M. grisea*	27%
ScSch9: *S.cerevisiae* (kinase-like protein)	19%
CaSch9: *C. albicans* (kinase-like protein)	19%
AnSchA: *A. nidulans* (kinase-like protein)	15%

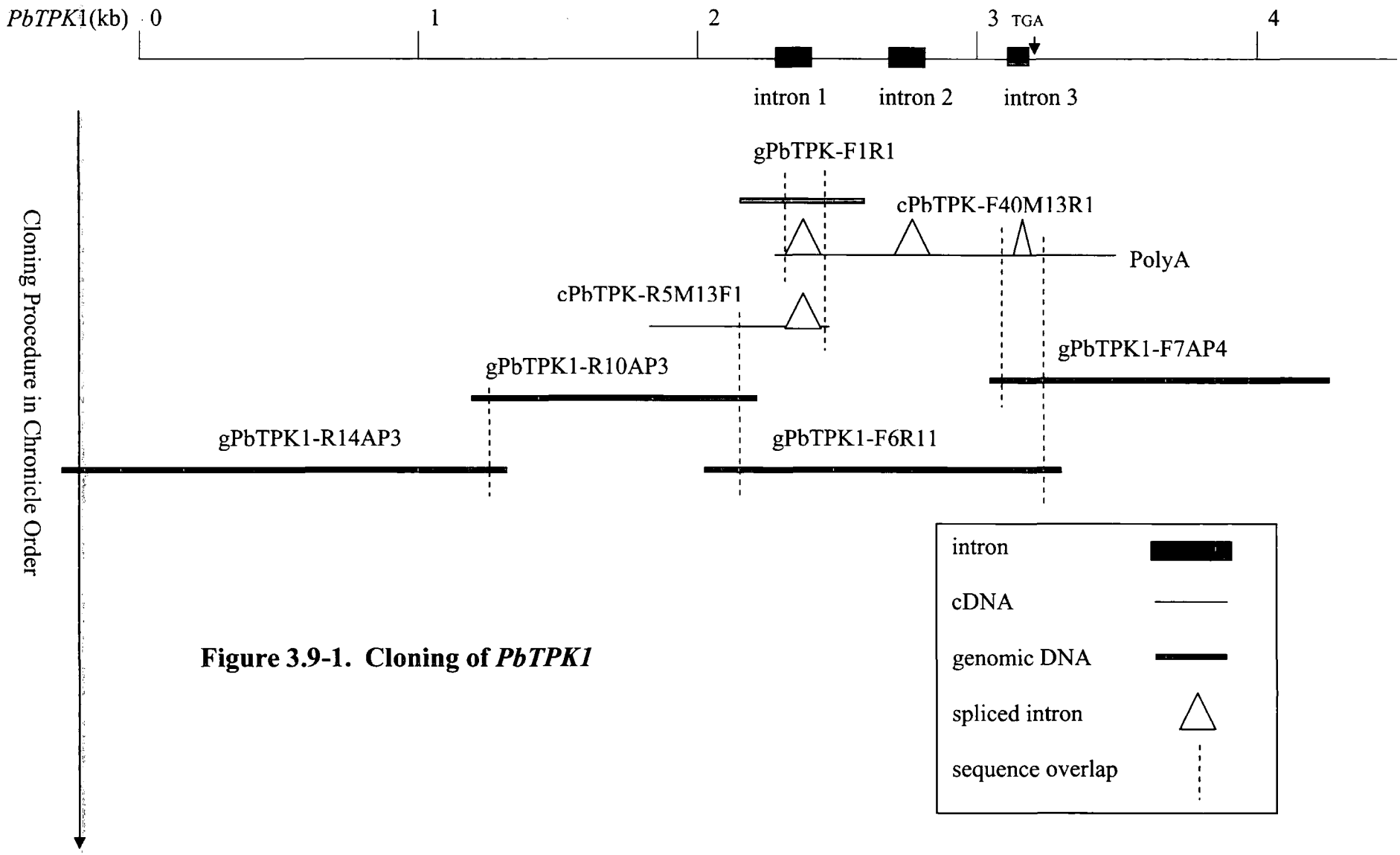


Figure 3.9-1. Cloning of *PbTPK1*

Figure 3.9-2. The nucleotide and deduced amino acid sequence of *PbTPKL1*. Nucleotide positions are written in red numerals, amino acids in blue. Introns are denoted by lowercase letters with red colour.

```
  1  CTCAAACTGAGGAGCCAATCGAATTCGAACTCGAGAATGGAACAAATGAG
  1     Q  T  E  E  P  I  E  F  E  L  E  N  G  T  N  E
 51  ACGCTATCATCTGTACCGATCCAACGATCCAACAGTGACTCAGGTCGCCC
 17    T  L  S  S  V  P  I  Q  R  S  N  S  D  S  G  R
101  CATGGCTATCCCAATGAAAAGTCGACAAAGTAGTAACAACAGCATGTTGG
 33  P  M  A  I  P  M  K  S  R  Q  S  S  N  N  S  M  L
151  ATAGTCATGGCCATAAGGGGCTAAGTGAGGTTACAGATCCATTGTGGAAT
 50   D  S  H  G  H  K  G  L  S  E  V  T  D  P  L  W  N
201  CATGAAGCCGTTTTgtgcgttttgcttcttttcttttctactcattatcc
 67    H  E  A  V
251  tttaattattggcaaagcctatgcgcgtccttatcttactgaccttatta

301  cttctagTGATGTCCTTGGGGATGAATCTGAAATTGAAGTTTCGGTATAC
 71        F  D  V  L  G  D  E  S  E  I  E  V  S  V  Y
351  GATCGATCGAACCACGAAGCGTTCTTGGGTCATGTAAAGTTGCGTGTCAG
 86    D  R  S  N  H  E  A  F  L  G  H  V  K  L  R  V
401  TTTTAAGGAAGATAATAAATCAATAGCAGGATGGTATCCTCTAGTGGTTC
102  S  F  K  E  D  N  K  S  I  A  G  W  Y  P  L  V  V
451  GTGATCGGGGTGATGGCTATGTGTCGGGAGAGATACATCTAGAAATGAGC
119   R  D  R  G  D  G  Y  V  S  G  E  I  H  L  E  M  S
501  TTTCAGAAAACAGACAAGAAGCAGTTTGGACCAAATGATTTCCAAATCCT
136    F  Q  K  T  D  K  K  Q  F  G  P  N  D  F  Q  I
551  TAAGCTCATAGGGAAGGGAACGTTTGGGCAAGTCTATCAAGTGAGGAAAA
152  L  K  L  I  G  K  G  T  F  G  Q  V  Y  Q  V  R  K
601  AGGACACCCAGAGAATCTACGCGATGAAAGTCCTTTCCAAAAAGGTGATC
169   K  D  T  Q  R  I  Y  A  M  K  V  L  S  K  K  V  I
651  ATTCAGAAAAAAGAAGTTGCCCATACACTTGGCGAAAGGAACATCCTGGT
176    I  Q  K  K  E  V  A  H  T  L  G  E  R  N  I  L
701  CCGGACTGCTATGACGAATTCTCCTTTCATAGTTGGTCTCAAATTTTCTT
202  V  R  T  A  M  T  N  S  P  F  I  V  G  L  K  F  S
751  TTCAAACACCCACAGATTTGTACCTTGTCACAGACTACATGTCTGGAGGG
218   F  Q  T  P  T  D  L  Y  L  V  T  D  Y  M  S  G  G
801  GAGCTGTTCTGGCATCTGCAAAAAGAAGGGAGATTTCAGGAAGCTAGAGC
236    E  L  F  W  H  L  Q  K  E  G  R  F  Q  E  A  R
851  GAAGTTTTACATCGCTGAGTTGATACTAGCTTTGCAGCATCTACACGAAC
252  A  K  F  Y  I  A  E  L  I  L  A  L  Q  H  L  H  E
901  ATGACATCGTGTATCGGGACCTCAAGCCAGAAAACATCCTACTTGATGCA
279   H  D  I  V  Y  R  D  L  K  P  E  N  I  L  L  D  A
951  AATGGTCACATTGCGCTCTGCGATTTTGGTCTATCCAAGGCAAAC
286    N  G  H  I  A  L  C  D  F  G  L  S  K  A  N
```

Figure 3.9-3. The nucleotide and deduced amino acid sequence of *PbTPK1*. Nucleotide positions are written in red numerals, amino acids in blue. Introns are denoted by lowercase letters with red colour. Polyadenylation site are underlined. The start codon does not include the Kozak sequence.

```
-2850   CAATCCTGGCATACCAAATAGAGAGCAAAAGGCACTAGAAATTGGGGCTG
-2800   TGGGACAAGCTGCAGGACCAGCAGCTTTCCAAAACTGGCTTCAGAAAGCT
-2750   GTATCCCCCCTCAATCTCATCACATATACTGATGGCTCCAAAGACAGTCA
-2700   AGGAGTTTCAGGCGGAGGCTACTGTCTATACTGAGGAACAAATCAATCTC
-2650   TGGAGCTTGGTAAGCTGCCTCTGGGCCACACTGCTGAGGTCTTTGATGCT
-2600   GAAGTGCTTGCAGCAGTTGCCAGTCTTGAGGCAGTGCTACGGAATCCCTT
-2550   CTCTATGTTCACACAGCACATTATAGTGGCTCTAGACAATGAAGAGGCTG
-2500   CAATAAGACTCCACACAGACATGCCCACACGAACATCTTTTCTCACCTTT
-2450   GCTAAGTTCAATGAACTCCACAGGTCCTGGACAAAACAGGGCCCAGGTCC
-2400   AGCCAGCCTGAGACGGGTCTATGTTCGATGGTGCCCCAGCCATGCTGGGA
-2350   TTCCAGGCAATGATGCTGCAGATGCTGCGGCCAAGTCAGCCTGCACAATG
-2300   GAATGCACTCACCGCACTCTGACCCTGGCACGGGCCAAATCCTATGCTAA
-2250   AGAACACTTCAATGCCTCACTGCAACAATACTGGAGCTCCAATAGCACAT
-2200   CACGATACAAGGACCTCAATATCCCAATGCCAAAAGGACCCTCCCCTGAG
-2150   CTCACTCTCCCCTGAAGGAACCTAGGCCACTTGCTGGCAGCTAGAACAGG
-2100   CCACAGTGACTTTGCAGCCTATCATCACAGATGGGCACATGACAATGCAC
-2050   TCCTCACCTGCTCTTGCGGCCGAGATAAGACCCCCGAGCACTTCTTCTTT
-2000   TGCTGGAAAGGCTGGAACATTGAATGAATTAAAACCCCTGAAGCATGCTG
-1950   AGGACCAAAGGAAGCAATTGACTGGCTTCTCAGCACAGTAAGAGGAGCTA
-1900   TGGCCTTCTCTACCTGGTGCACTAATACCCGCTTCTTCATCAACATCCAA
-1850   CGCTGCTACTAGAACCTGTTCAACATCCGACTTCTCCCTCCCCCTCTCTC
-1800   TTTCCTATGTTCCTTATATACATTTCTCATCACCCCTACACACCACCCTA
-1750   GAAGCCCTTGGCTTCCTCAGTCCCGTAATCTTCGGCTACTACGCCATTCA
-1700   GAACCTCCAGGACAGGGGCTACTCTGTACATAGACTCTCAAGAGGGCTTC
-1650   GGCCGACTAGACATGTCAGGCAGCAGACTAGCTGCTGCGCTGGCACGCCT
-1600   GATACCCTTAGGGAGGTGCTGTGGCCGCGACGGCAGACTATTCTTAGTCA
-1550   GCTGATCACGTGCCTCCAGGCGTTAAATAAGTAATAATAATAATAATAAT
-1500   AATAACTGGCTTATGCCCTAACTCGTCAGCCTGGTTAATGCCACTAAACA
-1450   ATCGGACTATTCTTTTTCCACCTGATAACTAAAATACATCCATAATACTT
-1400   CCACCGTTCATCTCTTCTTCCCTTAACTTCCCTAACTTCCCTCCCTTCTC
-1350   TCTCTACTCTCTTTTCTCTATTTTCTCTAATAACGAATTCGGGCGTGTTA
-1300   TGTAGGCAGTGCTAGAGCTGTATTCGGCAACGTGCCCATTCCAATAAGAA
-1250   AGATCATTATCACTTTTTACTCCTTCAAGCATGAGTAGAAGACCAGGAGC
-1200   TTATCGGTAAAAACGACCTTCGCGCTGAGTGTCTGCAGGTTCAATTCCAT
-1150   TCTACAGATGCCATACAACCCCCCAAAGCATAGTTAAGTCCTGTACGCAG
-1100   GTCGTCAAAAGCCAGATATCCGGAATTCTTCTTCATTAATCTCTGTCCAA
-1050   CAGCTCCCTGCAGAGCTCCTGCTGCCAATCCACAAGTCTCTCATGGTGCA
-1000   GGTGAATAAATTCAACCAACTACTGTCTTCTGAAATAACCATATATCTAT
 -950   ATAATCTATTCTAGCTAGTTATATTTTTAATGGTTTAACACATAATACGA
 -900   AAATATTATTGCGTTGCTGAGATGCCATTGAAAATATCCAGAGCTGGTAG
 -850   ACGAATTCATTGCGTAATAGAGTTTGTTGGGCAGCTGGATTAACTAACGT
 -800   TGCTATATATAGGTCAGAACAAAAAGCATAGTGATGCATATAACCCATCT
 -750   CTCTATCAATGTCTTCCTCTCGGTTAAAGAGTGCTGATACTAGGGCGAGA
 -700   TATTGTTCATAGGTTATGATGTAAGTTCGGGGTCCCTCTGTTCTGGGCGG
 -650   GACCACATACTCCGAGCAGGCACCTCATCCATAGTTATCTAATTCCCTTG
 -600   TTATAACTAACTATCTAGTGGCGTCGGGCCTCAGCATCATCGACGTCTGC
 -550   TTACTACAACGTCCCATTACTACCTCAACTACGGCCGTCATCTATTCCGA
 -500   ACGAGAGTACCAACAAACGTCAATCTTTGTCAAGTATTATAATTGCTCCC
 -450   CAGGCACCAGAATCAACGAATCAACCCCTCTACCATCGCCAATTCCTTTA
 -400   CCCTCCATTCCCCTGCCTCTCCGGTCCTGTCCATTCGTCCCGTCTACGCA
 -350   AATGCCTGCACTCGTTTGAACCTCACAGGTATCTGCTCCACCCTCCACAT
 -300   TAAGCACTCCCTCCGTACTTCGGATCCGTCTGTTTCCGACTATGTATTAA
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 -200   CCATCGCCCCCAACCAAGAATTTACCCACCCCTGAACCCCAAGGCTGCTG
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-150   TTAGATATCGGGAGCGTACCAACGCAAAAGGCCGTCGATAGATCATTTGA
-100   AGAAAATCAAACCATACCTCGAAACAACGTCTCTCTCATTGTCCTTGTCG
 -50   ATCCGTCATCCACCCACAGGCCTTGCCGTAGTCCCTTGTCTTGGCGTTCC
   1   ATGCGGGGTCTAGGCAATTTGCTGAAGAAGAAGAAGAAGCGGACGAAGGA
   1     M  R  G  L  G  N  L  L  K  K  K  K  K  R  T  K
  51   CTCGCGCATCTCGAGGGACCTGGATCTGTCCGCTTCGCCGGTAGCGGAGT
  17   D  S  R  I  S  R  D  L  D  L  S  A  S  P  V  A  E
 101   CAACCTCGTCGTCTCCTGGCGATAATAATAATAATATTATTAATAATAGT
  34    S  T  S  S  S  P  G  D  N  N  N  N  I  I  N  N  S
 151   AGCCCTCTCTTCCTGACGACAGCAGCTACAGCTACCGCACCAGCTACCGT
  51     S  P  L  F  L  T  T  A  A  T  A  T  A  P  A  T
 201   AACAGCAGCACCAACAACGAACACCCTGCTCCAGCACCCTGCGTTTACCT
  67   V  T  A  A  P  T  T  N  T  L  L  Q  H  P  A  F  T
 251   CACCCGCCCGCGATTCCTCTTGCAAAGACCCCGCTTCCTCCCCCGGCACC
  84    S  P  A  R  D  S  S  C  K  D  P  A  S  S  P  G  T
 301   AATAAGATCAGTAGCAGTAGTAATCCTCCTGCCTCCGTTTTCCTTACAGC
 101     N  K  I  S  S  S  S  N  P  P  A  S  V  F  L  T
 351   TATTCCTACCTCGACTAAACCAGAGGGAGTCGCCTCCTCTCCTCCCACTG
 117   A  I  P  T  S  T  K  P  E  G  V  A  S  S  P  P  T
 401   GCTCCACAACAGCTGGCGCAATCACGAAAATTGACAGCACACATTCTGCA
 134    G  S  T  T  A  G  A  I  T  K  I  D  S  T  H  S  A
 451   CCCATGAACCCCCTACATTCTCCCGGGCCCCCTTCCTCGGAATATAAGGC
 151     P  M  N  P  L  H  S  P  G  P  P  S  S  E  Y  K
 501   GGGCGATCCCTCTCATTTCAAACAGCACCAGCAACAGCAACAGCAACCAC
 167   A  G  D  P  S  H  F  K  Q  H  Q  Q  Q  Q  Q  Q  P
 551   AGCAGCAATCCCACCACTCCCAAAATGTCGCCAGTATTAAGAACATTATA
 184    Q  Q  Q  S  H  H  S  Q  N  V  A  S  I  K  N  I  I
 601   AATCCACCCCTCAACGATGAAGTGGCGGCCTCCCCACAACCAGTTGGCGA
 201     N  P  P  L  N  D  E  V  A  A  S  P  Q  P  V  G
 651   TGGCCTACATGCCGCACAACAACGAATGCATTCACAGCATCCACAGCAAC
 217   D  G  L  H  A  A  Q  Q  R  M  H  S  Q  H  P  Q  Q
 701   AACAACACCAACAGCAACAACAACAACAACAGCAACAACAACAACAACAA
 234    Q  Q  H  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q
 751   CAAGTTCAACAATCTGTCCCGTCAGTTTCGCGGCAGACAAAGGGCAAATA
 251     Q  V  Q  Q  S  V  P  S  V  S  R  Q  T  K  G  K
 801   CTCGCTAGATGACTTTACGTTGCAACGGACGTTGGGGACGGGCAGTTTCG
 267   Y  S  L  D  D  F  T  L  Q  R  T  L  G  T  G  S  F
 851   GGCGAGTACACCTGGTTCAATCGCGACATAACCAGCGCTTCTATGCCATC
 284    G  R  V  H  L  V  Q  S  R  H  N  Q  R  F  Y  A  I
 901   AAGGTGCTGAAGAAAGCGCAAGTGGTGAAGATGAAGCAGGTCGAGCATAC
 301     K  V  L  K  K  A  Q  V  V  K  M  K  Q  V  E  H
 951   GAATGATGAAAGAAGAATGCTGCAGCGGGTTAAACATCCATTCCTAATTA
 317   T  N  D  E  R  R  M  L  Q  R  V  K  H  P  F  L  I
1001   CGCTATGGGGCACGTTCCAGGATGTGAAGAATCTTTATATGGTTATGGAT
 334    T  L  W  G  T  F  Q  D  V  K  N  L  Y  M  V  M  D
1051   TTTGTGGAGGGTGGAGAGCTGTTTAGTTTACTGAGGAAATCGCAGgtggg
 351     F  V  E  G  G  E  L  F  S  L  L  R  K  S  Q
1101   gaaactccttttccttttccccttcggcttgttctatctaaagggatttttt

1151   ttttgaactaattgatatcggttcctcagCGCTTCCCAAATCCTGTTGCA
 366                                 R  F  P  N  P  V  A
1201   AAGTTCTACGCTGCGGAGGTTACGTTGGCGTTGGAATATCTACATGACCA
 373     K  F  Y  A  A  E  V  T  L  A  L  E  Y  L  H  D
1251   TCACATTATATACCGAGACCTAAAGCCAGAGAATCTACTTTTAGATCGAT
 389   H  H  I  I  Y  R  D  L  K  P  E  N  L  L  L  D  R
1301   ATGGACATTTGAAGATTACAGATTTTGGATTCGCGAAGGAAGTCAAGGAT
 406    Y  G  H  L  K  I  T  D  F  G  F  A  K  E  V  K  D
1351   ATTACATGGACTTTATGCGGGACACCAGATTATCTGGCGCCGGAGGTAGT
 423     I  T  W  T  L  C  G  T  P  D  Y  L  A  P  E  V
1401   TTCGTCAAAGGGATACAACATGTCCGTTGACTGgtaagcggttctccgcc
 439   V  S  S  K  G  Y  N  M  S  V  D
```

```
1451   ccccccccccccccccctcctttttatttttatttttatttttgtattg

1501   ttccaccgcattacacaccactaaccactccactccataatcaaccgaaa

1551   agGTGGTCCCTCGGCATCCTAATCTTCGAAATGCTTTGCGGCTTCACCCC
 450     W  W  S  L  G  I  L  I  F  E  M  L  C  G  F  T
1601   CTTCTGGGACGGCGGCAGCCCCATGAAAATCTACGAAAACATCATAAAAT
 466   P  F  W  D  G  G  S  P  M  K  I  Y  E  N  I  I  K
1651   GCCGAGTCAAGTATCCGTCCTACATGCACCCGGACGCCCAAGATCTACTC
 483    C  R  V  K  Y  P  S  Y  M  H  P  D  A  Q  D  L  L
1701   TCCCAACTCATTACTCCCGATCTCACAGTACGCCTGGGCAACTTGCACGG
 500     S  Q  L  I  T  P  D  L  T  V  R  L  G  N  L  H
1751   CGGCAGTAAGGATGTTAAGGATCATCCGTGGTTTGCGGAGGTGACGTGGG
 516   G  G  S  K  D  V  K  D  H  P  W  F  A  E  V  T  W
1801   ATCGGCTAGCGCGGAAGGATATTGATGCGCCGTATATTCCTCCGGTGAAG
 533    D  R  L  A  R  K  D  I  D  A  P  Y  I  P  P  V  K
1851   GGAGGGCAGGGGGATGCAAGCCAGTTTGATAGGTATCCCGAGGAGACGGA
 550     G  G  Q  G  D  A  S  Q  F  D  R  Y  P  E  E  T
1901   GCCGTATGGGGGTAGTGGGGATGATCCgtatgtttctccttgctttccct
 566   E  P  Y  G  G  S  G  D  D
1951   ctcgtttgcttcaacgacgaagctttactaatgatgttatagATATGGCG
 575                                                 P  Y  G
2001   ATTATTTCGTGGACTTTTGAATGGGGATAATTTTGTGAAGACCCGTCGAC
 578    D  Y  F  V  D  F  *
2051   TGCGCTCTCTAGGTATTTCTATATATCTACCGGCCTATGCCAATATCTAG

2101   ACGGAGAATTTTTATATGACCAATCATCCGCCTTTACATATTTCTTTTTC
2151   CATTCTAAAAAGGGTCGGGGAAAAAACGAACTGAAAAGGAAAAGAAGGGA
2201   CGGGAAACGATGTTTTATTCAATTCTCTCTCTTGAAGATAAATGTATGCA
2251   TTTCCATCCGTATTAACGGGTTCTCTTCTTCTACTCTGAGAAAATATACG
2301   GCCTTGGCCTGTATCTTCTAGCTTTTTGGGCCTCCCATTTCCTTTGGGCC
2351   CTCATTTTCATCATTTAACTCTATCTTGCTCCATGGGACGGTTATACCTG
2401   AGACTAACAGCGGGGTCTGTGAAAGGATTTATTTATTTTGTCCTTGCTAC
2451   TCCATACTTGTTTTTTGAGTTCTCTTAACTATCATATACGGATTTCTATT
2501   TTCGTACTTAATCTTTCTTTTCTTCTTTTCTTTCTCGTTCATTGGGGGAG
2551   TGTTTAATATTATTGGACTTTTGTTTGGGGATGACATGTTCTTCTCAGAG
2601   TATCTTTTTGGTCAATGGAATGGGCTTGCTATGGCGATCCGTGACATAAA
2651   ATCAGTGAGTTGATGTAATGGGAAATGAAAATGCTGTGAAAAGAGAAGAT
2701   AAACGTTTTGTTTCCCTATGGTCATGTATTTCATATCTTCTACCGTTTTT
2751   GTCTCTCTTTTCTCGTTCATCGTACGTCGCTTATTCCTGGATCCTACATA
2801   GTCGTCGTGAATAGATTTTATAGCCCGACTTAATAGTCAGACTTAAAATA
2851   AGAAAAGGGGTGTTCATATCTGTTCCTTAATTTCTCAATTCATACTTGGT
2901   ATCCTAACTTTTGTCATGGATGATAGAAACCATTCCTTCGTGTGATTCAC
2951   CGCTCCCATGTTTCAGAAAAGGGGCAGGATAGGAAAAATGGAAATAGGAC
3001   TGGAAAAGCATAAACTATAAATACAGGGAGATAAAAACAAGACCACCCAG
3051   TAGGAGGTTATAGGGAGCAGGTGCGTGCAAAAAGGGGGGGGAAAAGCGAG
3101   GAGATATATAAA
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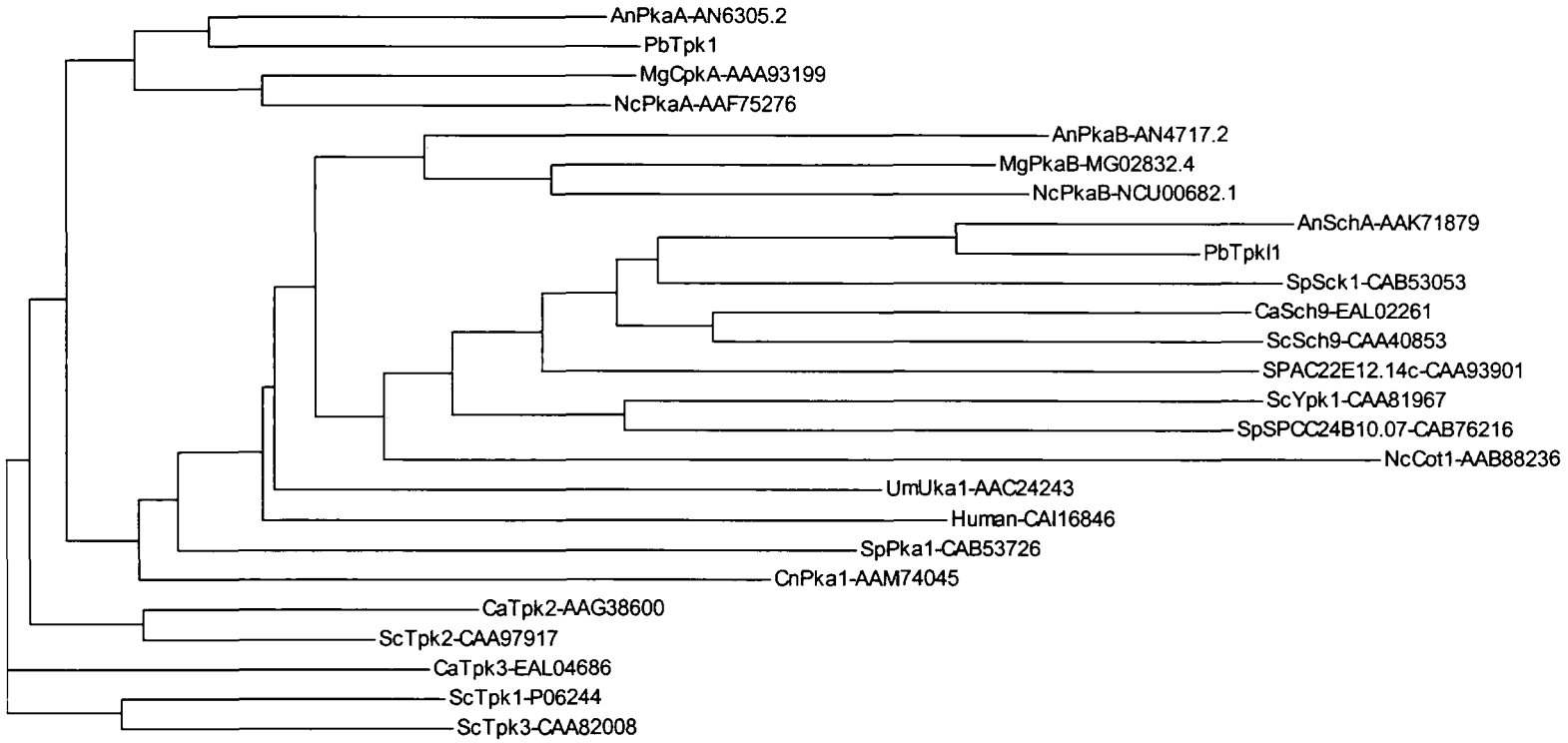
Figure 3.9-4. Phylogenetic analysis of fungal cAMP-dependent protein kinase catalytic subunits and cAMP-dependent protein kinases-like proteins. The tree was created with Vector NTI 6.0 (Informax). Protein GenBank accession numbers are given after the name of the protein. The abbreviations are: Pb, *P. brasiliensis*; An, *A. nidulans*; Ca, *Candida albicans*; Nc, *Neurospora crassa*; Mg, *Magnaporthe grisea*; Um, *Ustilago maydis*; Cn, *Cryptococcus neoformans*; Sc, *S. cerevisiea*; and Sp, *S. pombe*.



3.10 Cloning a Transcription Repressor Gene *PbTUPA* from *Pb01*

Cloning of *PbTUPA* was achieved by a degenerate PCR approach and amplifications with SSP-RCR from both our *Pb01* genomic DNA and cDNA libraries. Two degenerate primers, PbTUPA-F1 and PbTUPA-R1, were used to clone a fragment of the gene with genomic DNA. Specific primers were then designed on the basis of the sequence of this fragment (see Table 2.6-1); and with these primers, fragments of the genomic DNA and cDNA were cloned and sequenced. By overlapping the sequences, the full-length gene was delineated, as illustrated in Figure 3.10-1.

The coding sequence of *PbTUPA*, which is interrupted by 7 introns, is 1,776 bp in length and encodes a protein of 592 amino acids with a molecular weight of 65 kDa and predicted pI of 6.4. Like other fungal TupA proteins, the PbTupA protein contains a predicted N-terminal coiled-coil domain and seven WD40 domains (Figure 3.10-2). It is highly conserved with PmTupA (GenBank No. AAL99251; 79% identity), AnTupA (GenBank No. AAG28504; 74% identity), NcRco1 (GenBank No. P78706; 58% identity), SpTup1 (GenBank No. CAB52736; 40% identity) and ScTup1 (GenBank No. CAA42259; 35% identity), as illustrated in Figure 3.10-2. The predicted site of initiation of translation of *PbTUPA* appears correct because the position of the first methionine is conserved with that of *P. marneffei* TupA (Figure 3.10-2). However, the first ATG of *PbTUPA* cDNA does not meet the requirement of a Kozak sequence (ANNATGG). It is worth noting that *A. nidulans* TupA has an extra 26 amino acids beyond the N-terminal ends of both PbTupA and PmTupA (Figure 3.10-2), suggesting the N-terminal ends of PbTupA and PmTupA are incomplete. Another possibility is that *Pb* can use an alternative consensus sequence at the site of translation initiation. It requires further

Figure 3.10-1. The nucleotide and deduced amino acid sequence of *PbTUPA*. Nucleotide positions are written in red numerals, amino acids in blue. Introns are denoted by lowercase letters with red colour.

```
-100   ACCGCAGCTCTCTTCCTGTCTTCCTTTCAGACAGTGCAACTCAAATAATC
 -50   AACATAACTTCTTACCTTAGGCCAGCCTCGCCCTCAAGGCTTGTGCCAAC

   1   ATGTATAACCCACACCGTGGTATGGTTACTGCTCCAAACTCACGATTGAC
   1     M  Y  N  P  H  R  G  M  V  T  A  P  N  S  R  L
  51   AGAGCTGCTCGATCAGCTGCGCCAGGAGTATGAGAACCAGTCGCGGAATA
  17   T  E  L  L  D  Q  L  R  Q  E  Y  E  N  Q  S  R  N
 101   CCGGCGAGTTTGAACATCAACgtgtgttcccccctcccccctctttcttt
  34    T  G  E  F  E  H  Q
 151   ctttcttccttccttccttttttcttcttcttataataccctcccaaccct
 201   tatctcggaaacaggtgtagccatctctctgtcatgtctgcaattacaat
 251   agacgcaacgtgcatatgaacgcatggttttgcgcccgatgttgtcatca
 301   aataccccctttttgcacactgttaaccgttcactgacaaacttttgcat
 351   ttgcattcacagTCACTGGCCAACTCCAGGAGATGGAGATGATCAGACAG
  41                L  T  G  Q  L  Q  E  M  E  M  I  R  Q
 401   AAAGTGTACCAGCTGGAGCAGGCGCAGATAAAAATGAAGCAAGAgtacgt
  54     K  V  Y  Q  L  E  Q  A  Q  I  K  M  K  Q
 451   cttcggctcttatttataatcctcataaatgatctcgagtgagagtgttt
 501   gctaactgtctctgcacttgctttcaagATACGAGGCTGAAATCCGCACT
  68                                  E  Y  E  A  E  I  R  T
 551   CTTCGAATGGAACTCGAATCTCGTGGCGTGCATCTCCCCCAGTCTCACAT
  75     L  R  M  E  L  E  S  R  G  V  H  L  P  Q  S  H
 601   TGGTGGTCCCCCAACACATGCTGGGCCTTCTCAAGCTCCTCCTCCCGCGC
  92   I  G  G  P  P  T  H  A  G  P  S  Q  A  P  P  P  A
 651   TTGGACATGGCCCTGCCAATCTGTTTGGCGGCATCATGACCAACCCAGGA
 109    L  G  H  G  P  A  N  L  F  G  G  I  M  T  N  P  G
 701   GGTGGAGCTCCAGCCCTTGCGCCCCCTCCTCCTCCACAGGACCAGCAACA
 125     G  G  A  P  A  L  A  P  P  P  P  P  Q  D  Q  Q
 751   ACCGCCCCAGCACGCTCTTCAGCAACCCGGTTCTGGCGCCCAAGGCCCTC
 142   Q  P  P  Q  H  A  L  Q  Q  P  G  S  G  A  Q  G  P
 801   CTCAACCACAGCAAGGTGCCTTTGGAGGGTATCAGCCTGCCTCTGCAGTT
 158    P  Q  P  Q  Q  G  A  F  G  G  Y  Q  P  A  S  A  V
 851   AACGgtatgtcccaacgtcacaaaatatgggaaaagcaaaattctgatca
 176     N
 901   aaactgttatagGTTATGGACCTCCACCTCCCGCTCCAACCTCGTCCCCA
 177                G  Y  G  P  P  P  P  A  P  T  S  S  P
 951   GGCCCTGGAAAAGGCAGAGCCAGTCGCGCACCTCCCGGTCCAGCCACCCC
 190     G  P  G  K  G  R  A  S  R  A  P  P  G  P  A  T
1001   CCAACAGAACCATCAAGTCGCCTATCCCGACACTCGTGCATCCCCTCAGA
 206   P  Q  Q  N  H  Q  V  A  Y  P  D  T  R  A  S  P  Q
1051   TGCCCCGCCCCACTCCTCCCGGCCAGCAACTGAGCTATCGCGTAGGAAAC
 223    M  P  R  P  T  P  P  G  Q  Q  L  S  Y  R  V  G  N
1101   ACACTCGCAGACCTGGATCCTGACAAGTTGCCCCCCAGCCAAAAGCGCGA
 240     T  L  A  D  L  D  P  D  K  L  P  P  S  Q  K  R
1151   AGGTTCTGATTGGTACGCCGTTTTCAACCCGGAGGTTCCTCGTTTGCTGG
 256   E  G  S  D  W  Y  A  V  F  N  P  E  V  P  R  L  L
1201   ATGTCGACCTTGTCCACCATCTCGTTCATGACAGTGTAGTTTGTTGTGTT
 273    D  V  D  L  V  H  H  L  V  H  D  S  V  V  C  C  V
1251   AGGTTCAGCAATGATGGGAAATTTGTTGCTACGGGCTGCAATCGCTCTGC
 290     R  F  S  N  D  G  K  F  V  A  T  G  C  N  R  S
1301   GCAGATCTTTGACGTTCAGAGTGGACAGCTTGTCACCTCTTTGCAAGATG
 306   A  Q  I  F  D  V  Q  S  G  Q  L  V  T  S  L  Q  D
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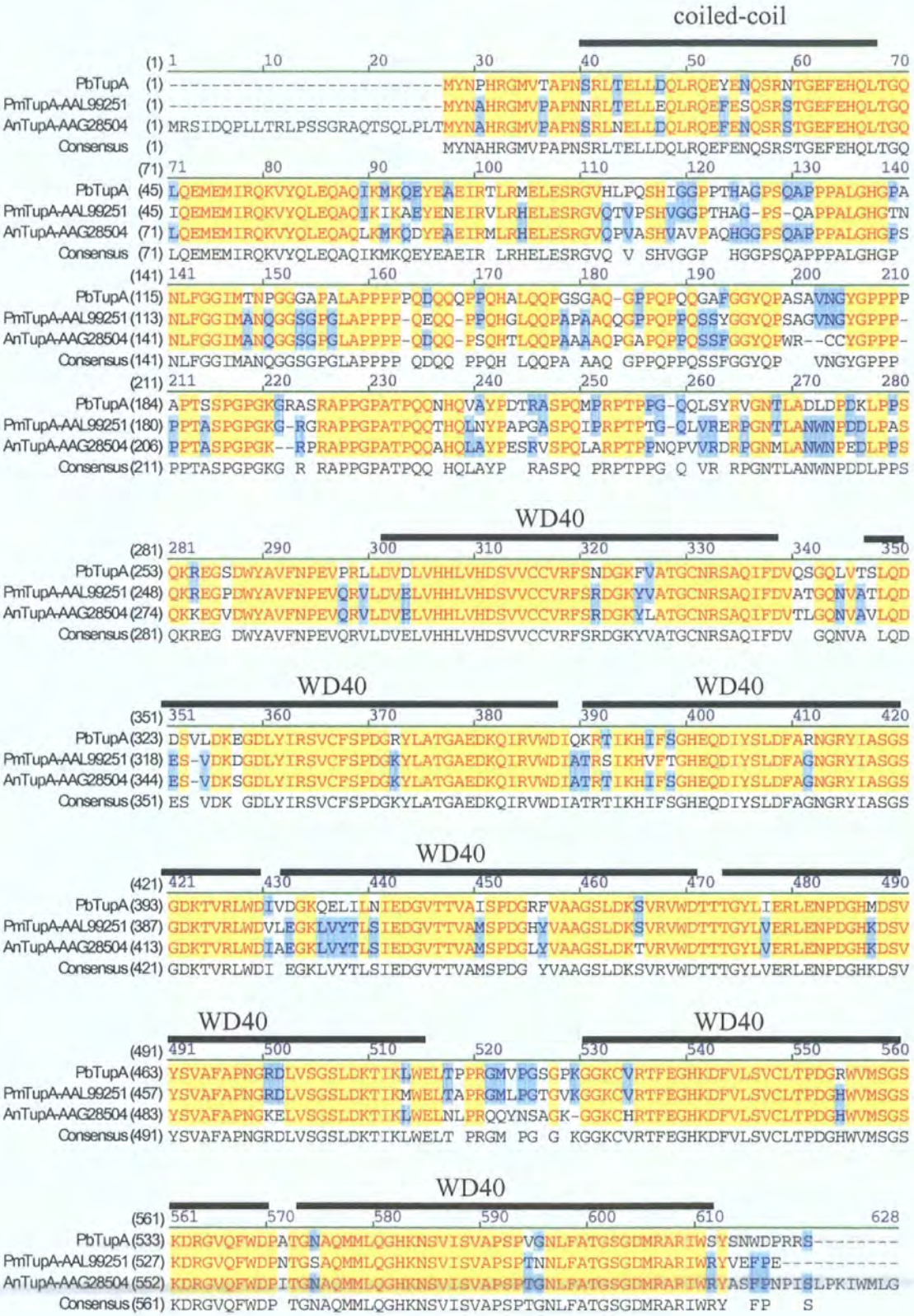
1351  ATTCTGTTCTTGATAAGGAAGGGGACCTTTATATCCGTAGCGTCTGCTTT
 323   D  S  V  L  D  K  E  G  D  L  Y  I  R  S  V  C  F
1401  AGTCCAGATGGGAGATACCTTGCTACTGGTGCAGAAGATAAACAGATTAG
 340    S  P  D  G  R  Y  L  A  T  G  A  E  D  K  Q  I
1451  Agtaaggacatgcccttgttctttttctttctgctatttatatattgtt
 356  R
1501  ccattccaccaactaacctccacctacagGTCTGGGATATCCAAAAACGC
 357                                 V  W  D  I  Q  K  R
1551  ACCATAAAACACATCTTCTCCGGCCACGAACAAGATATCTACTCTCTCGA
 364    T  I  K  H  I  F  S  G  H  E  Q  D  I  Y  S  L
1601  CTTCGCCCGCAATGGCCGCTACATTGCCTCCGGCTCCGGCGACAAAACCG
 379  D  F  A  R  N  G  R  Y  I  A  S  G  S  G  D  K  T
1651  TCCGCCTTTGGGACATCGTCGACGGCAAGCAGGAACTAATCCTCAACATC
 397   V  R  L  W  D  I  V  D  G  K  Q  E  L  I  L  N  I
1701  GAAGATGGCGTTACTACCGTCGCCATATCTCCCGATGGCCGCTTCGTCGC
 414    E  D  G  V  T  T  V  A  I  S  P  D  G  R  F  V
1751  TGCCGGTTCCCTCGATAAGAGTGTTCGGGTCTGGGATACGACGACTGGAT
 430  A  A  G  S  L  D  K  S  V  R  V  W  D  T  T  T  G
1801  ATCTTATTGAGCGTCTTGAGAATCCGGATGGGCATATGGATAGTGTGTAT
 447   Y  L  I  E  R  L  E  N  P  D  G  H  M  D  S  V  Y
1851  TCTGTGGCGTTTGCGCCGAATGGGAGGGATCTTGTGTCGGGCAGTTTGGA
 464    S  V  A  F  A  P  N  G  R  D  L  V  S  G  S  L
1901  TAAGACTATTAAATTGTGGGAGTTGACGCCGCCTAGAGGGATGGTCCCTG
 479  D  K  T  I  K  L  W  E  L  T  P  P  R  G  M  V  P
1951  GTTCTGGTCCTAAGGGTGGCAAGTGTGTTAGGACTTTTGAGGGGCATAAG
 497   G  S  G  P  K  G  G  K  C  V  R  T  F  E  G  H  K
2001  gtgggtttcccctctttttgtgtcaattcaagttgatgaaggctgtaact
2051  gacatatctatgtttcttcgcagGATTTTGTTCTTAGTGTCTGCTTGACA
 514                          D  F  V  L  S  V  C  L  T
2101  CCCGATGGACGCTGGGTTATGAGTGGATCTAAGGATCGTGGCGTACAGTT
 523    P  D  G  R  W  V  M  S  G  S  K  D  R  G  V  Q
2151  CTGGGATCCAGCGACTGGGAATGCGCAGATGATGCTTCAGGGCCATAAGA
 539  F  W  D  P  A  T  G  N  A  Q  M  M  L  Q  G  H  K
2201  ATTCAGgttcgttattcgtttttattctttgtttctgcggccccctcct
 556   N  S
2251  tttgtctctcaagctaaccatatcctccttcgttagTTATCTCCGTCGCA
 558                                       V  I  S  V  A
2301  CCCAGCCCAGTCGGCAACCTGTTTGCGACCGGAAGCGGAGACATGCGTGC
 563    P  S  P  V  G  N  L  F  A  T  G  S  G  D  M  R
2351  TAGAATCTGGAGgttagtttcaaccctcaacgtttgatatcatatatttg
 579  A  R  I  W
2401  cgtttctgttcatcctcgttatcaccgccgtcatcatataattcgcgctc
2451  ccttgccccttctaccatagtttgtctttgtatccactcttatttattct
2501  ccttcatgtcccagtatactaacgtctacaaaacctaactctcgacatgg
2551  gatttacagCTATTCCAATTGGGATCCCAGAAGGTCATAGGTTATGTTTA
 583           S  Y  S  N  W  D  P  R  R  S  *
2601  ATTCACAAGCGGACGCAGGCACGCTCACGCATATGGCAGAACTTGCAAAA
2651  CATGCAGTTGGGCATATATTTAAATATGCGCAACACGTAACTTTGCCAGA
2701  GGAAGGAAATAAAGGAGGAAACGGGCCAAGGGAAAGGGGAAAGGCGTTCT
2751  GTGGCTACTACATTTACCGGCTCTTGCGATTGATTGG
```

Figure 3.10-2. PbTupA is highly conserved with TupA from *P. marneffei* and *A. nidulans*. Alignment was done with Vector NTI 6.0. GenBank protein accession numbers are indicated after the name of protein. The predicted N-terminal coiled-coil domain and seven WD40 repeats are shown with black lines.



amplification of upstream cDNA fragments to turn this hypothesis into a conclusion.

3.11 Plasmid Constructions for Yeast-Two-Hybrid Analysis

As illustrated in Figure 3.5-1 and Figure 3.5-2, a fidelity *PbCYR1* cDNA has been cloned into vectors to obtain the constructions pYES6-cPbAC-F26R34-1-14 (-21 bp cDNA version with a point mutation Q600R), pBSK-cPbAC-F26R34-1 (full-length cDNA version) and pGEMTE-cPbAC-F26R34-1 (full-length cDNA version) as templates for downstream sub-cloning. Fidelity full-length cDNAs of *PbGPA1*, *PbGPA2*, *PbGPA3*, *GPR1* of *S. cerevisiae* (*ScGPR1*) and the actin gene of *Pb* (*PbACTIN*, GenBank accession No. AY383732) have also been cloned into pGEM T Easy vector as templates for sub-cloning (Table 3.11). These cDNAs were then sub-cloned into Clontech's bait vector pGBKT7 and prey vector pGADT7 to make constructions for Yeast-2-Hybrid experiments. All the genes in the constructions are in frame with the vectors. Some constructions of *PbRAS1* (*RAS* gene of *Pb*, GenBank accession No. AY547438; cloned by Dr M.I. Borges-Walmsley) and *PbGPB1* were made by Dr M.I. Borges-Walmsley and Dr G. Chen in our lab. Major constructions in this thesis are illustrated in the following Table 3.11.

Table 3.11 Major plasmid constructions used in this thesis.

Plasmid Names	Vectors	Cloning and insert descriptions
Constructions as templates		
pYES6-cPbAC-F26R34-1-14	pYES6/CT (Invitrogen)	See Figure 3.5-1; The insert is the -21 bp *PbCYR1* cDNA version with a point mutation Q600R in addition to the mutation A2S introduced by primer PbAC-F26.
pBSK-cPbAC-F26R34-1	pBSK (Stratagene)	See Figure 3.5-2; The insert is the full-length *PbCYR1* cDNA version without any point mutation at protein level

		except for the mutation A2S introduced by primer PbAC-F26.
pGEMTE-cPbAC-F26R34-1	pGEM T Easy (Promega)	See Figure 3.5-2; The insert is the full-length *PbCYR1* cDNA version without any point mutation at protein level except for the mutation A2S introduced by primer PbAC-F26.
pGEMTE-cPbGPA1-ExGPAF1pYER3-9(T7>SP6)	pGEM T Easy (Promega)	The insert is the RT-PCR product amplified with PbGPA1-ExGPAF1 and PbGPA1-pYER3 from *Pb01* mRNA. The insert is the full-length *PbGPA1* cDNA.
pGEMTE-cPbGPA2-F6R6-2(SP6>T7)	pGEM T Easy (Promega)	The insert is the RT-PCR product amplified with PbGPA2-F6 and PbGPA2-R6 from *Pb01* mRNA. The insert is the full-length *PbGPA2* cDNA.
pGEMTE-cPbGPA3-F5R5-2(T7>SP6)	pGEM T Easy (Promega)	The insert is the RT-PCR product amplified with PbGPA3-F5 and PbGPA3-R5 from pDNR-*Pb01*cDNA library. The insert is the full-length *PbGPA3* cDNA.
pGEM-TE-PbACTIN-GADF1R1-12(SP6>T7)	pGEM T Easy (Promega)	The insert is RT-PCR product amplified with PbActinGADF1 and PbActinGADR1 from *Pb01* mRNA. The insert is the full-length *PbACTIN* cDNA.
pGEMTE-ScGPR1-F1R1-17	pGEM T Easy (Promega)	The insert is the full length *ScGPR1* which was cloned by recombination of fidelity PCR products. The sequence of *ScGPR1* in this clone spans from the position of primer ScGPR1-F1 to that of ScGPR1-R1.
Constructions for Y2H		
pGAD- *PbCYR1*$^{1-678}$	pGADT7 (Clontech)	*PbCYR1*$^{1-678}$ fragment from pGBK-*PbCYR1*$^{1-678}$ was digested with NdeI/BamHI and cloned into pGADT7 (NdeI/BamHI). This insert corresponds to the RA domain of PbCyr1.
pGAD- *PbCYR1*$^{600-1316}$	pGADT7 (Clontech)	*PbCYR1*$^{600-1316}$ fragment from pGBK-*PbCYR1*$^{600-1316}$ was digested with NdeI/BamHI and cloned into pGADT7 (NdeI/BamHI). This insert corresponds to the LRR domains of PbCyr1.

pGAD- *PbCYR1*$^{1302\text{-}1876}$	pGADT7 (Clontech)	*PbCYR1* cDNA fragment (NdeI/XhoI digested) obtained by PCR with PbAC-EF1 and PbAC-ER2 was cloned into pGADT7 (NdeI/XhoI). This insert corresponds to the PP2Cc and CYCc domains of PbCyr1.
pGAD- *PbCYR1*$^{1648\text{-}2100}$	pGADT7 (Clontech)	*PbCYR1* cDNA fragment (NdeI/XhoI digested) obtained by PCR with PbAC-EF2 and PbAC-ER1 was cloned into pGADT7 (NdeI/XhoI). This insert corresponds to the CYCc domain of PbCyr1.
pGAD-*PbGPA1*	pGADT7 (Clontech)	Full-length *PbGPA1* insert from pGBK-*PbGPA1* was digested with NdeI and cloned into pGADT7 (NdeI).
pGAD-*PbGPA2*	pGADT7 (Clontech)	Full-length *PbGPA2* insert from pGBK-*PbGPA2* was digested with NdeI/SalI and cloned into pGADT7 (NdeI/XhoI).
pGAD-*PbGPA3*	pGADT7 (Clontech)	Full-length *PbGPA3* insert from pGBK-*PbGPA3* (NdeI/BamHI) was cloned into pGADT7 (NdeI/BamHI).
pGAD-*PbGPB1*	pGADT7 (Clontech)	Full-length *PbGPB1* cDNA (NdeI/BamHI) was cloned into the vector pGADT7 (NdeI/BamHI); This construction was made by Dr G. Chen in our lab.
pGAD-*PbRAS1*	pGADT7 (Clontech)	Full-length *PbRAS1* insert from pGBK-*PbRAS1* (NdeI/EcoRI) was cloned into pGADT7 (NdeI/EcoRI).
pGAD-*PbRAS1*$^{1\text{-}83}$	pGADT7 (Clontech)	PCR product amplified with 5'BD Screening and PbRAS-R1 as primers, and pGBK-*PbRAS1* as template was digested with NdeI/EcoRI and cloned into pGADT7 (NdeI/EcoRI).
pGAD-*PbRAS1*$^{26\text{-}238}$	pGADT7 (Clontech)	PCR product amplified with 3'BD Screening and PbRAS-F1 as primers, and pGBK-*PbRAS1* as template was digested with NdeI/EcoRI and cloned into pGADT7 (NdeI/EcoRI).
pGAD-*PbACTIN*	pGADT7 (Clontech)	Full-length *PbACTIN* insert (NdeI/EcoRI) from pGEMTE-PbACTIN-GADF1R1-12 was cloned into pGADT7 (NdeI/EcoRI).
pGAD-*PbACTIN*$^{1\text{-}314}$	pGADT7	PCR product amplified with PbACTIN-

	(Clontech)	R3 and SP6 as primers and pGEMTE-PbACTIN-GADF1R1-12 as template was digested with NdeI/EcoRI and cloned into pGADT7 (NdeI/EcoRI).
pGAD-*ScGPR1*$^{679\text{-}961}$-F5R1	pGADT7 (Clontech)	PCR product amplified with ScGPR1-F5 and ScGPR1-R1 as primers and pGEMTE-ScGPR1-F1R1-17 as template was digested with BamHI and cloned into pGADT7 (BamHI); This insert is the C-terminal cytoplasmic domain of *ScGPR1*.
pGAD-*ScGPR1*$^{274\text{-}621}$-F4R3	pGADT7 (Clontech)	PCR product amplified with ScGPR1-F4 and ScGPR1-R3 as primers and pGEMTE-ScGPR1-F1R1-17 as template, was digested with BamHI and cloned into pGADT7 (BamHI); This insert is the third cytoplasmic loop of *ScGPR1*.
pGAD-*ScGPR1*$^{1\text{-}961}$-F3R1	pGADT7 (Clontech)	PCR product amplified with ScGPR1-F3 and ScGPR1-R1 as primers, and pGEMTE-ScGPR1-F1R1-17 as template, was digested with BamHI and cloned into pGADT7 (BamHI); This insert is the full-length *ScGPR1*.
pGADT7-T	pGADT7 (Clontech)	From Clontech
pGAD-*P53*$^{72\text{-}390}$	pGADT7 (Clontech)	P53 insert from pGBKT7-P53 (NdeI/BamHI digested), was cloned into pGADT7 (NdeI/BamHI).
pGAD-*lam*$^{66\text{-}230}$	pGADT7 (Clontech)	Lam insert from pGBKT7-Lam (NdeI/BamHI digested) was cloned into pGADT7 (NdeI/BamHI).
pGBK-*PbCYR1*$^{1\text{-}678}$	pGBKT7 (Clontech)	*PbCYR1* cDNA fragment obtained by PCR with PbAC-GADF1 and PbAC-GADR1 was digested with NcoI/BamHI and cloned into pGBKT7 (NcoI/BamHI). This insert corresponds to the RA domain of PbCyr1.
pGBK-*PbCYR1*$^{600\text{-}1316}$	pGBKT7 (Clontech)	*PbCYR1* cDNA fragment obtained by PCR with PbAC-GADF2 and PbAC-GADR2 was digested with NcoI/BamHI and cloned into pGBKT7 (NcoI/BamHI). This insert corresponds

		to the LRR domain of PbCyr1.
pGBK-*PbCYR1*$^{1302\text{-}1876}$	pGBKT7 (Clontech)	*PbCYR1* cDNA fragment obtained by PCR with PbAC-EF1 and PbAC-ER2 was digested with NdeI/XhoI and cloned into pGBKT7 (NdeI/SalI). This insert corresponds to the PP2Cc and CYCc domain of PbCyr1.
pGBK-*PbCYR1*$^{1648\text{-}2100}$	pGBKT7 (Clontech)	*PbCYR1* cDNA fragment obtained by PCR with PbAC-EF2 and PbAC-ER1 was digested with NdeI/XhoI and cloned into pGBKT7 (NdeI/SalI). This insert corresponds to the CYCc domain of PbCyr1.
pGBK-*PbGPA1*	pGBKT7 (Clontech)	Full-length cDNA of *PbGPA1* from pGEMTE-cPbGPA1-ExGPAF1pYER3-9 was digested with NdeI and cloned into pGBKT7 (NdeI).
pGBK-*PbGPA2*	pGBKT7 (Clontech)	PCR product obtained by PCR with PbGPA2-F7 and T7 as primers and pGEMTE-cPbGPA2-F6R6-2 as template was digested with EcoRI and cloned into pGBKT7 (EcoRI). The insert is the full-length cDNA.
pGBK-*PbGPA3*	pGBKT7 (Clontech)	PCR product obtained by PCR with PbGPA3-F8 and SP6 as primers and pGEMTE-cPbGPA3-F5R5-2 as template was digested with EcoRI and cloned into pGBKT7 (EcoRI digested). The insert is the full-length cDNA.
pGBK-*PbGPB1*	pGBKT7 (Clontech)	Full-length *PbGPB1* cDNA (PCR product digested with NdeI/BamHI) was cloned into the vector pGBKT7 (NdeI/BamHI); This construction was made by Dr G. Chen in our lab.
pGBK-*PbRAS1*	pGBKT7 (Clontech)	Full-length *PbRAS1* cDNA (PCR product digested with NdeI/EcoRI) was cloned into the vector pGBKT7 (NdeI/EcoRI); This clone was made by Dr M.I. Borges-Walmsley.
pGBKT7-53	pGBKT7 (Clontech)	From Clontech
pGBKT7-Lam	pGBKT7 (Clontech)	From Clontech
pGBK-*ScGPR1*$^{1\text{-}961}$-F3R1	pGBKT7	Insert (BamHI digested) from pGAD-

	(Clontech)	*ScGPR1*$^{1\text{-}961}$-F3R1 was cloned into pGBKT7 (BamHI). This insert is the full-length *ScGPR1*.
pGBK-*ScGPA2*-F1R1	pGBKT7 (Clontech)	PCR product obtained with ScGPA2-F1 and ScGPA2-R1 as primers and *S. cerevisiae* DNA as template was digested with BamHI and cloned into pGBKT7 (BamHI); This insert is the full-length *ScGPA2*.

3.12 Construction of Random Mutagenesis Libraries for Yeast-Two-Hybrid Screening

Nine random mutagenesis libraries were made with Stratagene's GeneMorph II Random Mutagenesis kit and cloned into prey vector pGADT7 for yeast-2-hybrid screening. These libraries were so named as the following: pGAD-*PbGPA1*-RM-Lib, pGAD-*PbGPA2*-RM-Lib, pGAD-*PbGPA3*-RM-Lib, pGAD-*PbGPB1*-RM-Lib, pGAD-*PbRAS1*-RM-Lib, pGAD-*PbACTIN*-RM-Lib, pGAD-*ScGPR1*$^{679\text{-}961}$-RM-Lib, pGAD-*P53*-RM-Lib, and pGAD-*lam*-RM-Lib. The procedures for the individual constructions are described below.

For pGAD-*PbGPA1*-RM-Lib, random mutagenesis PCR was undertaken using 5'BD Screening and 3'BD Screening as primers, and pGBKT7-*PbGPA1* as template. The PCR product was cleaned with Qiagen's Gel Extraction column, digested with *Nde*I/*Sal*I, and ligated into pGADT7 (*Nde*I/*Xho*I). The ligation product was transformed into XL-10 Gold Ultracompetent Cell (Stratagene), generating 4 X 10^4 transformants. Five clones were randomly selected and sequenced for quality control of the library. The plasmid library pGAD-*PbGPA1*-RM-Lib was obtained with Qiagen's plamid Maxi kit from pooled transformants. The final concentration of this library was 2.4 μg/μl of plasmid DNA.

For pGAD-*PbGPA2*-RM-Lib, random mutagenesis PCR was undertaken using PbGPA2-F10 and 3'BD Screening as primers, and pGBKT7-*PbGPA2* as template. The PCR product was cleaned, digested with *Nde*I/*Eco*RI, and ligated into pGADT7 (*Nde*I/*Eco*RI). The ligation product was transformed into XL-10 Gold Ultracompetent Cell, generating 4 X 10^4 transformants. Five clones were randomly selected and sequenced for quality control of the library. The plasmid library pGAD-*PbGPA2*-RM-Lib was obtained with Qiagen's plamid Maxi kit from pooled transformants. The final concentration of this library was 2.2 μg/μl of plasmid DNA.

For pGAD-*PbGPA3*-RM-Lib, random mutagenesis PCR was undertaken using PbGPA3-F11 and 3'BD Screening as primers, and pGBKT7-*PbGPA3* as template. The PCR product was cleaned, digested with *Nde*I/*Eco*RI, and ligated into pGADT7 (*Nde*I/*Eco*RI). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 4 X 10^4transformants. Five clones were randomly selected and sequenced for quality control of the library. The plasmid library pGAD-*PbGPA3*-RM-Lib was obtained with Qiagen's plamid Maxi kit from pooled transformants. The final concentration of this library was 2.0 μg/μl of plasmid DNA.

For pGAD-*PbGPB1*-RM-Lib, random mutagenesis PCR was undertaken using 5'BD Screening and 3'BD Screening as primers, and pGBKT7-*PbGPB1* as template. The PCR product was cleaned, digested with *Nde*I/*Bam*HI, and ligated into pGADT7 (*Nde*I/*Bam*HI). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 4 X 10^4transformants. The plasmid library pGAD-*PbGPB1*-RM-Lib was obtained with Qiagen's plamid Maxi kit from pooled transformants. The final

concentration of this library was 1.7 μg/μl of plasmid DNA.

For pGAD-*PbRAS1*-RM-Lib, random mutagenesis PCR was underatken using 5'BD Screening and 3'BD Screening as primers, and pGBKT7-*PbRAS* as template. The PCR products were pooled, cleaned, digested with *Nde*I/*Eco*RI, and ligated into pGADT7 (*Nde*I/*Eco*RI). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 4 X 10^4transformants. The plasmid library pGAD-*PbRAS1*-RM-Lib was obtained with Qiagen's plamid Maxi kit from pooled transformants. The final concentration of this library was 1.7 μg/μl of plasmid DNA.

For pGAD-*PbACTIN*-RM-Lib, random mutagenesis PCR was underatken using PbACTIN-GADF2 and T7 as primers, and pGEMTE-*PbACTIN*-GADF1R1-12 as template. The PCR product was cleaned, digested with NdeI/EcoRI, and ligated into pGADT7 (*Nde*I/*Eco*RI). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 2 X 10^4transformants. The plasmid library pGAD-*PbACTIN*-RM-Lib was obtained with Qiagen's plamid Maxi kit from pooled transformants. The final concentration of this library was 0.4 μg/μl of plasmid DNA.

For pGAD-*ScGPR1*$^{679\text{-}961}$-RM-Lib, random mutagenesis PCR was undertaken using 5'AD Screening and 3'AD Screening as primers, and pGAD-*ScGPR1*$^{679\text{-}961}$-F5R1 (C-terminal cytoplasmic domain) as template. PCR product was cleaned, digested with *Nde*I/*Xho*I, and ligated into pGADT7 (*Nde*I/*Xho*I). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 2 X 10^4transformants. The plasmid library pGAD-*ScGPR1*$^{679\text{-}961}$-RM-Lib was obtained with Qiagen's plamid Maxi kit from pooled transformants. The final concentration of this library

was 0.25 μg/μl of plasmid DNA.

For pGAD-*P53*-RM-Lib, random mutagenesis PCR was undertaken using 5'BD Screening and 3'BD Screening as primers, and pGBK-53 as template. The PCR products was cleaned, digested with *Nde*I/*Bam*HI, and ligated into pGADT7 (*Nde*I/*Bam*HI). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 4 X 10^4transformants. The plasmid library pGAD-*P53*-RM-Lib was obtained with Qiagen's plamid Maxi kit from pooled transformants. The final concentration of this library was 0.4 μg/μl of plasmid DNA.

For pGAD-*Lam*-RM-Lib, random mutagenesis PCR was undertaken using 5'BD Screening and 3'BD Screening as primers, and pGBK-*Lam* as template. The PCR product was cleaned, digested with *Nde*I/*Bam*HI, and ligated into pGADT7 (*Nde*I/*Bam*HI). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 4 X 10^4transformants. The plasmid library pGAD-*lam*-RM-Lib was obtained with Qiagen's plamid Maxi kit from pooled transformants. The final concentration of this library was 0.4 μg/μl of plasmid DNA.

The Random mutagenesis libraries are summarized as below in Table 3.12.

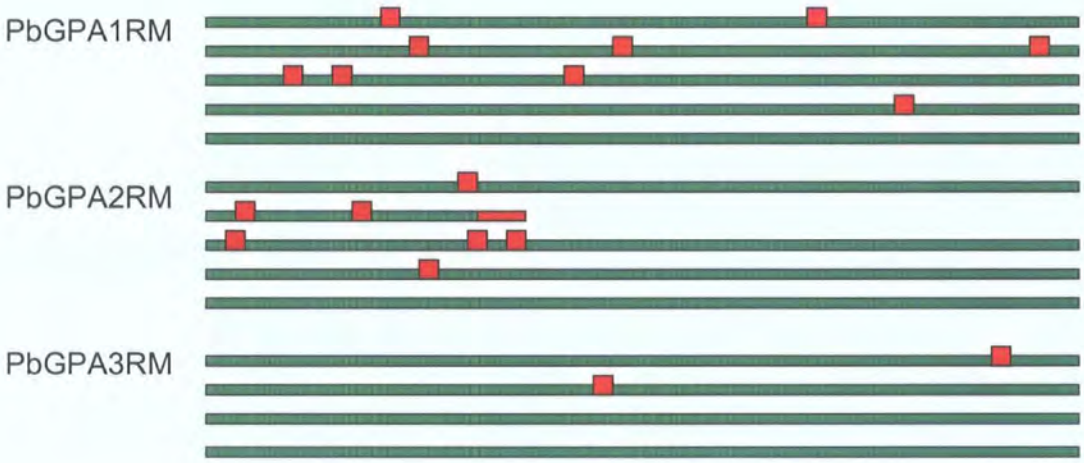
Table 3.12. Random Mutagenesis Libraries.

RM libraries	Original titer	Concentration of library plasmids
pGAD-*PbGPA1*-RM-Lib	$4X10^4$	2.4 μg/μl
pGAD-*PbGPA2*-RM-Lib	$4X10^4$	2.2 μg/μl
pGAD-*PbGPA3*-RM-Lib	$4X10^4$	2.0 μg/μl
pGAD-*PbGPB1*-RM-Lib	$4X10^4$	1.7 μg/μl
pGAD-*PbRAS1*-RM-Lib	$4X10^4$	1.7 μg/μl
pGAD-*PbACTIN*-RM-Lib	$2X10^4$	0.4 μg/μl

pGAD-*ScGPR1*$^{679\text{-}961}$-RM-Lib	$2X10^4$	0.25 μg/μl
pGAD-*P53*-RM-Lib	$4X10^4$	0.4 μg/μl
pGAD-*lam*-RM-Lib	$4X10^4$	0.5 μg/μl

Quality control sequencing of the libraries indicated that the mutations introduced by the GeneMorphII Enzyme (Stratagene) are evenly distributed and there is no hot spot for clustering of mutations (Figure 3.12).

Figure 3.12. Quality control of random mutagenesis libraries. Three random mutagenesis libraries were selected for quality test, with 5 clones from each library undergone for DNA sequencing. The upper panel gives a schematic demonstration of mutations created. DNA strands are indicated by green lines. Mutations are indicated by red blocks. The lower table gives the detail of mutations. Mutations are evenly distributed along the DNA without obvious hot spots of point mutations, frame shifts and terminations.



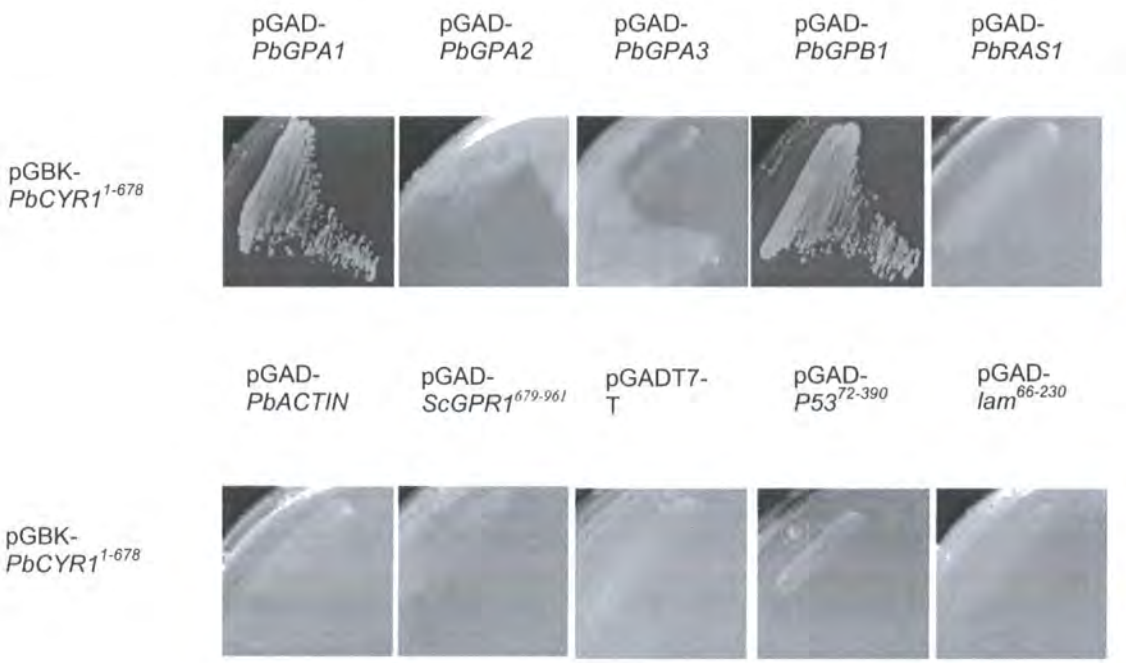
Random Mutagenesis Libraries	Mutations (5 clones tested for each library)
pGAD-PbGPA1-RM-Lib	E238D; Q98K, G202D, R348G; S47P, E65G, L194H; No mutation at amino acid level; E291V
pGAD-PbGPA2-RM-Lib	D119V; K14E, H61Q, mis-sense mutations from 86-97, Δ98-359; No mutation; D10E, P116S, E122K; L110P
pGAD-PbGPA3-RM-Lib	R265N; P141L; No mutation; No mutation; One sequencing reaction failed

3.13 Full-Length PbGpa1 and PbGpb1 Interact with PbCyr$^{1-678}$ in the Yeast-Two-Hybrid System.

As indicated in Table 3.11, segments *PbCYR1*$^{1-678}$, *PbCYR1*$^{600-1316}$, *PbCYR1*$^{1302-1876}$ and *PbCYR1*$^{1648-2100}$ were cloned into the bait vector pGBKT7, while full-length *PbGPA1*, *PbGPA2*, *PbGPA3*, *PbGPB1* and *PbRAS1* were cloned into the prey vector pGADT7. Each bait construction was then combined with each prey construction to undergo simultaneous transformation in yeast-2-hybrid analyses. The results indicated that pGAD-*PbGPA1* and pGAD-*PbGPB1* directly interact with pGBK-*PbCYR1*$^{1-678}$ because cotransformants with these 2 combinations of bait and prey construction grew well in SD/-Ade/-His/-Leu/-Try, while cotransfromants with other combinations could not grow on SD/-Ade/-His/-Leu/-Try (Figure 3.13).

In order to exclude the possibility of false positives, 6 control constructions were used. As indicated in Table 3.11, full-length *Pb* actin gene, and *S. cerecisiae GPR1* C-terminal cytoplasmic domain were cloned into pGADT7 to make constructions pGAD-*PbACTIN*, and pGAD-*ScGPR1*$^{679-961}$-F5R1. Fragments of the P53 and Lamin C genes were removed from pGBKT7-53 and pGBKT7-Lam (both from Clontech) to make constructions pGAD-*P53*$^{72-390}$ and pGAD-*lam*$^{66-230}$. Clontech's pGADT7 (empty vector) and pGADT7-T (SV40 large T antigen) were directly used without modification in yeast-2-hybrid analyses. The results indicated that all 6 aforementioned prey constructions could not interact with pGBK-*PbCYR1*$^{1-678}$ (Figure 3.13). Therefore, the following conclusion was drawn: full-length PbGpa1 and PbGpb1 interact with the first third of *Pb* adenylate cyclase PbCyr1$^{1-678}$ (the Ras association domain is included in this region) in the yeast-2-hybrid system, while full-length PbGpa2, PbGpa3, PbRas1 and PbActin do not interact with PbCyr1 in the

Figure 3.13. Full-length PbGpa1 and PbGpb1 directly interact with PbCyr1$^{1-678}$. Bait and prey vectors were simultaneously transformed into yeast strain AH109 and plated out on SD/-Leu/-Trp for 3 days. Yeast colonies grown on SD/-Leu/Trp were re-streaked on SD/-Ade/-His/-Leu/-Trp and incubated for 3 days. Results shown below indicate that pGAD-*PbGPA1* and pGAD-*PbGPB1* can directly interact with pGBK-*PbCYR1*$^{1-678}$, while full-length pGAD-*PbGPA2*, pGAD-*PbGPA3*, pGAD-*PbRAS1*, and pGAD-*PbACTIN* can not directly interact with pGBK-*PbCYR1*$^{1-678}$.



	pGBK-*PbCYR1*$^{1-678}$	pGBK-*PbCYR1*$^{600-1316}$	pGBK-*PbCYR1*$^{1302-1876}$	pGBK-*PbCYR1*$^{1648-2100}$
pGAD-*PbGPA1*	+	-	-	-
pGAD-*PbGPA2*	-	-	-	-
pGAD-*PbGPA3*	-	-	-	-
pGAD-*PbGPB1*	+	-	-	-
pGAD-*PbRAS1*	-	-	-	-
pGAD-*PbACTIN*	-	Not tested	Not tested	Not tested
pGAD-*ScGPR1*$^{679-961}$	-	Not tested	Not tested	Not tested
pGADT7-T	-	Not tested	Not tested	Not tested
pGAD-*P53*$^{72-390}$	-	Not tested	Not tested	Not tested
pGAD-*lam*$^{66-230}$	-	Not tested	Not tested	Not tested
pGADT7	-	Not tested	Not tested	Not tested

yeast-2-hybrid system.

3.14 Screening Random Mutagenesis (RM) Libraries: Truncated Versions of PbGpa2, PbGpa3, PbRas1, and PbActin Interact with PbCyr1$^{1-678}$

Given that full-length PbRas1 does not interact with adenylate cyclase PbCyr1 (Figure 3.13); a hypothesis was made that either an additional iso-form of Ras exists in *Pb* with adenylate cyclase binding activity, or PbRas requires assistance of cognate partner(s) to interact with adenylate cyclase. Previous reports indicate that Ras directly acts on adenylate cyclase in *S. cerevisiae* (Shima et al, 1997; Kido et al, 2002). Besides, there is an obvious Ras association domain in PbCyr1. However, only one *RAS* gene has been cloned form *Pb* even though extensive degenerate PCR primers have been used (personal communication with Dr M.I. Borges-Walmsley). A further question was raised for *PbGPA2* and *PbGPA3*: if they do not interact with *PbCYR1*, what do they interact with? With bait constructions pGBK-*PbGPA2* and pGBK-*PbGPA3*, a *Pb01* cDNA library in prey vector pGADT7 was screened in a search for PbGpa2 and PbGpa3 interacting partners (this job was done by Dr G. Chen in our lab). Several candidate genes were obtained and their functions require further analysis (data not shown). However, these genes seem not to belong to the existing cAMP signalling and MAP kinase pathway. With the belief in mind that PbGpa2, PbGpa3 and PbRas1 still work on PbCyr1 but they may switch between inactive and active binding states *in vivo* in *Pb* cells, which are unresolved in *S. cerevisiae* yeast two-hybrid experiments, random mutagenesis libraries (Table 3.12) of these genes were made and used in screening for mutants that were locked in an active dominant form to interact with PbCyr1. The library screening turned out to be successful. The four bait constructions pGBK-*PbCYR1*$^{1-678}$; pGBK-*PbCYR1*$^{600-1316}$;

pGBK-*PbCYR1*$^{1302\text{-}1876}$ and pGBK-*PbCYR1*$^{1648\text{-}2100}$ were used in screening. Positive clones were obtained from each RM library of *PbGPA1*, *PbGPA2*, *PbGPA3*, *PbGPB1*, and *PbRAS1* as illustrated in Table 3.14-1.

Table 3.14-1. Number of positive clones obtained after screening random mutagenesis (RM) libraries with truncated constructions of PbCyr1.

RM Libraries	Pre-transformed Bait Vector			
	pGBK-*PbCYR1*$^{1\text{-}678}$	pGBK-*PbCYR1*$^{600\text{-}1316}$	pGBK-*PbCYR1*$^{1302\text{-}1876}$	pGBK-*PbCYR1*$^{1648\text{-}2100}$
pGAD-*PbGPA1*-RM-Lib	≈200	0	0	0
pGAD-*PbGPA2*-RM-Lib	55	0	0	0
pGAD-*PbGPA3*-RM-Lib	8	0	0	0
pGAD-*PbGPB1*-RM-Lib	≈100	0	0	0
pGAD-*PbRAS1*-RM-Lib	3	0	0	0

It is interesting to observe that all positive clones arose from screening with pGBK-*PbCYR1*$^{1\text{-}678}$, indicating that the first third of adenylate cyclase is the region for protein interaction. In fact, pGAD-*PbGPA1*-RM-Lib and pGAD-*PbGPB1*-RM-Lib were used as positive controls because we had already proved that *PbGPA1* and *PbGPB1* interact with *PbCYR1*$^{1\text{-}678}$ (Figure 3.13). About 200 and 100 positive clones were obtained respectively from screening the 2 positive control libraries. Given that the transformation efficiency was about 10^5-10^6 per library screening, 100-200 positive clones were only a very small portion of all transformants, indicating that most mutations had disrupted the structure of the protein with a consequent loss of interaction. Meanwhile, it was interesting to determine what kind of mutations arose in *PbGPA2*, *PbGPA3* and *PbRAS1* to promote interaction with PbCyr1$^{1\text{-}678}$. Prey plasmid constructions were retrieved and purified from positive

clones after screening and submitted for sequence analysis. Sequencing results disclosed that these mutations are truncations as demonstrated in Table 3.14-2.

Table 3.14-2. Sequencing results of positive clones obtained from screening mutagenesis libraries with pGBK-*PbCYR1*$^{1-678}$. Note: clones were selected randomly before sequencing analysis.

RM Libraries	**Sequences of positive clones after screening with pGBK-*PbCYR1*$^{1-678}$**
pGBK-*PbGPA1*-RM-Lib	L91P, F199T. Δ211-353, K54N. Δ209-353, E65D, I107T. Δ160-353, L38M. Δ157-353. Δ155-353. Δ145-353. Δ139-353.
pGBK-*PbGPB1*-RM-Lib	K229E, S257T. K323L. Δ319-353, missense mutations 307-318. Δ314-353, F219L, A227G. Δ304-353, P205S, V292D. Δ257-353. Δ223-353, L90P. Δ221-353, R107T, A220C. Δ180-353, A12E, S108P.
pGBK-*PbGPA2*-RM-Lib	Δ200-359, missense mutations 184-199. Δ184-359. Δ174-359. Δ171-359, missense mutations 153-170. Δ107-359, missense mutations 103-106. Δ103-359. Δ103-359, G63D.
pGBK-*PbGPA3*-RM-Lib	V76L. Δ214-356. Δ198-356. Δ192-356, S30T. Δ189-356, V127A. Δ175-356, S7Y. Δ164-356. Δ161-356.
pGBK-*PbRAS1*-RM-Lib	Q74R, G157W, V179D, R205I. Δ1-25, L107I. Δ149-238, missense mutations 84-148.

The results of Table 3.14-2 are also schematically illustrated in Figure 3.14-1. As indicated in the table and the figure, it was surprising to find that most positive clones turned out to be truncated versions of the genes after screening. This was significant because no propensity for truncation has been found in any original RM libraries. As aforementioned, full-length PbGpa1 and PbGpb1 but not full-length PbGpa2, PbGpa3 and PbRas1 interact with PbCyr1$^{1-678}$. It was not surprising that some point mutations and C-terminal truncations still retain their ability to interact. In this experiment, the shortest version for PbGpa1 was PbGpa1$^{1-138}$, and PbGpb1$^{1-179}$ for PbGpb1. For PbGpa2, all the mutants sequenced were C-terminal truncated (some with further point mutation or frame-shifts that caused stretches of missense mutations). It is worth noting that 3 mutants (Δ184-359, Δ174-359, Δ103-359) are only C-terminal truncations without any further mutations. For PbGpa3, only one mutant of those sequenced was the full-length gene form (with one point mutation V76L). Others were C-terminal truncated versions. It is worth noting that 4 clones were C-terminal truncations without any other forms of mutations (i.e. Δ214-356; Δ198-356; Δ164-356; and Δ161-356).

For *PbRAS1*, only 3 positive clones were obtained after screening, which were all sequenced. One clone was the full-length *PbRAS1* with 4 point mutations (Q74R, G157W, V179D, and R205I). It is worth noting that the other 2 clones were truncations, one N-terminal truncation with an additional point mutation (Δ1-25+L107I) and the other, a C-terminal truncation with a stretch of mutations caused by a frame-shift (Δ149-238+missense mutations 84-148). It is interesting that the N-terminal deletion was obtained from screening of the RM library, although this phenomenon is not fully understood. To exclude the possibility that the interactions

were caused by the point mutations rather than truncations, 2 new prey constructions (pGAD-*PbRAS1*$^{1-83}$ and pGAD-*PbRAS1*$^{26-238}$ as indicated in Table 3.11) were made, which retained the truncations but eliminated the point mutations. Yeast-2-hybrid results indicated that pGAD-*PbRAS1*$^{1-83}$ and pGAD-*PbRAS1*$^{26-238}$ can interact with *PbCYR1*$^{1-678}$, confirming that truncations without any additional mutation lead to interaction with *PbCYR1*$^{1-678}$.

Apart from the aforementioned results, it was surprising to find that a C-terminal truncated version of *Pb* actin PbActin could also interact with PbCyr1$^{1-678}$. Initially, the actin gene *PbACTIN* was used as a negative control in RM library screening. An RM library (pGAD-*PbACTIN*-RM-Lib) was made (Table 3.12) and screened with pGBK-*PbCYR1*$^{1-678}$. About 200 positive clones were obtained on SD/-Ade/-His/-Leu/-Trp media. Four randomly selected clones were used for sequencing. The results indicated that these clones fell into 3 types of C-terminal truncations as shown below.

RM librariy	**Sequencing results of 4 positive clones after screening pGAD-*PbACTIN*-RM-Lib**
pGAD-*PbACTIN*-RM-Lib	Δ325-375, D244V, and missense mutations 315-324. Δ327-375, I10N, Q59H, V76A, R206H, and K326R. Δ336-375, R116P, L189F, missense mutations 316-335.

In order to exclude the possibility that the interaction was caused by the point mutations rather than the C-terminal truncations, a new construction pGAD-*PbACTIN*$^{1-314}$ was made as described in Table 3.11. Sequencing confirmed that the construction pGAD-*PbACTIN*$^{1-314}$ had no other mutations except for the truncation Δ315-375. Yeast-2-hybrid analysis indicated that this truncated version of *Pb* actin interacts with PbCyr1$^{1-678}$.

To exclude the possibility of false positives, 3 negative control RM libraries were made by recruiting 3 genes, i.e. *ScGPR1*$^{679\text{-}961}$ (C-terminal cytoplasmic domain, functional as indicated by interacting with *ScGPA2*), P53 and Lamin C, because they do not interact with *PbCYR1*$^{1\text{-}678}$ (Table 3.12 and Figure 3.13). No positive clones were obtained after screening RM library pGAD- *ScGPR1*$^{679\text{-}961}$-RM-Lib. However, about 1,000 and 300 positive clones were obtained respectively after screening pGAD-*P53*-RM-Lib and pGAD-*lam*-RM-Lib. Sequencing results indicated that none of these clones contained any forms of truncations. However, point mutations are essential for their interaction with *PbCYR1*$^{1\text{-}678}$ (Figure 3.14-2). It is worth noting that for Lamin C, a mutation from E or D to V at the end of the gene seems essential for its interaction with PbCyr1$^{1\text{-}678}$. It still requires further investigation that point mutation(s) of an unrelated gene such as P53 or Lamin C can lead to the interaction with PbCyr1$^{1\text{-}678}$. In this thesis, my understanding of this issue is that interactions caused by point mutation(s) but not by truncations without point mutation are considered false positives, especially for genes that are not closely related to the cAMP signaling pathway.

In general, truncated versions of PbGpa2, PbGpa3, PbRas1 and PbActin can interact with PbCyr1$^{1\text{-}678}$ as shown in Figure 3.14-3.

Figure 3.14-1. Sequencing results of positive clones obtained from screening mutagenesis libraries with pGBK-*PbCYR1*$^{1-678}$. This is the schematic diagram of the table 3.14-2. Clones were randomly selected before sequencing analysis. Genes were denoted by green lines. Mutations are indicated by red blocks.

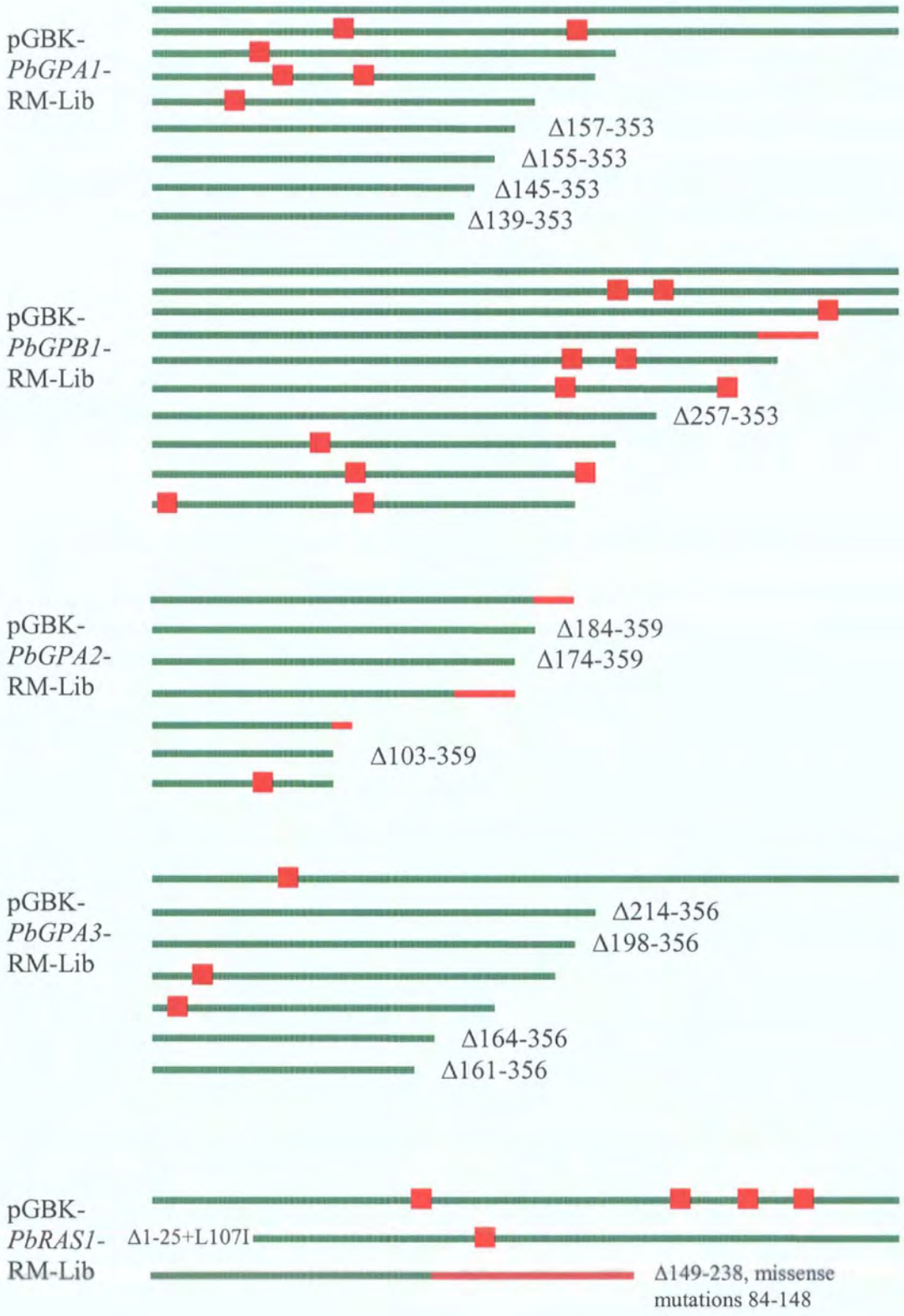


Figure 3.14-2. Sequencing results of positive clones obtained from screening negative control mutagenesis libraries with pGBK-*PbCYR1*$^{1-678}$. Clones were randomly selected before sequencing analysis. Genes were denoted by green lines. Mutations are indicated by red blocks. Mutated P53 and Lam genes were obtained after screening. No positive clones were obtained after screening random mutagenesis library pGAD-*ScGPR1*$^{679-961}$-RM-Lib.

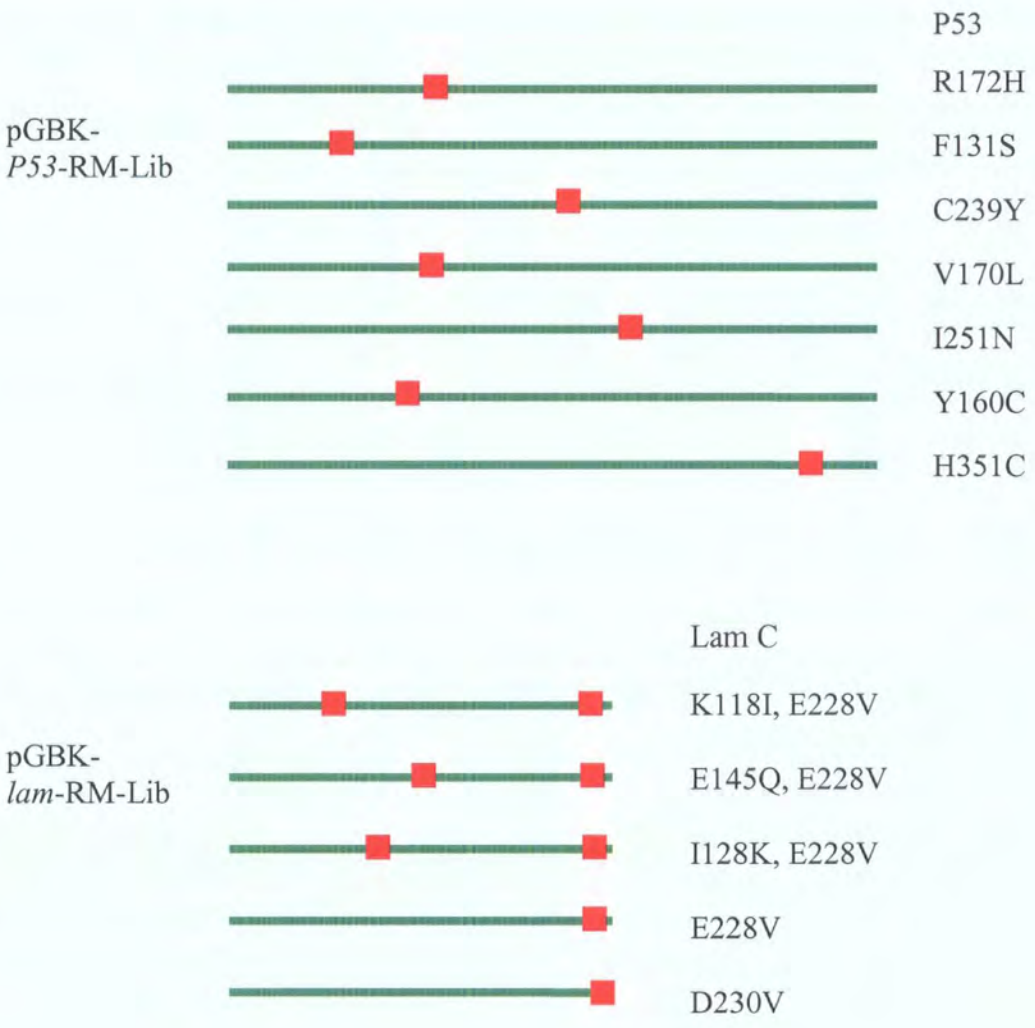
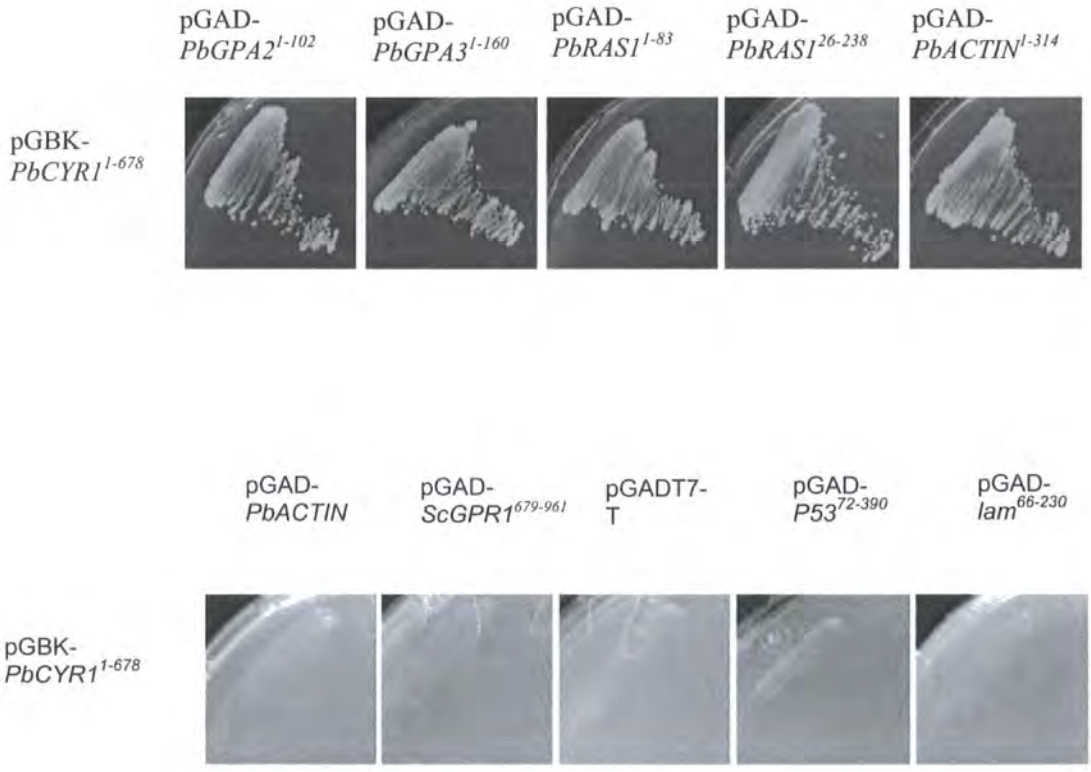


Figure 3.14-3. Truncated versions of PbGpa2, PbGpa3, PbRas1 and PbActin interact with PbCyr1$^{1-678}$. Bait and prey vectors were simultaneously transformed into yeast strain AH109 and plated out on SD/-Leu/-Trp for 3 days. Yeast colonies grown on SD/-Leu/Trp were re-streaked on SD/-Ade/-His/-Leu/-Trp and incubated for 3 days. Results shown below indicate that pGAD-*PbGPA2*$^{1-102}$, pGAD-*PbGPA3*$^{1-160}$, pGAD-*PbRAS1*$^{1-83}$, pGAD-*PbRAS1*$^{26-238}$ and pGAD-*PbACTIN*$^{1-314}$ can interact with pGBK-*PbCYR1*$^{1-678}$, while controls pGAD-*PbACTIN*, pGAD-*ScGPR1*$^{679-961}$, pGADT7-T, pGAD-*P53*$^{72-390}$, and pGAD-*lam*$^{66-230}$ can not directly interact with pGBK-*PbCYR1*$^{1-678}$.



3.15 Yeast-Two-Hybrid: Some Negative Results

In *S. cerevisiae*, *S. pombe* and *C. albicans* GPRs are membrane proteins, for which their cytoplasmic sides interact with a G protein α subunit and their cell surface side senses signalling molecules (Xue et al, 1998; Lamaire et al, 2004; Hoffman, 2005b; Miwa et al, 2004; Maidan et al, 2005b). We supposed that *GPR* exists in *Pb* and this gene could be conserved with other fungal *GPRs*, with a capacity to interact with our cloned *Pb* G protein α subunits. However, no *GPR* homologue was obtained when screening a *Pb01* cDNA library with pGBK-*PbGPA1*, *2*, and *3*. Another pilot experiment was done by screening RM library pGAD-*ScGPR1*$^{679\text{-}961}$-RM-Lib with pGBK-*PbGPA1*, *2*, and *3*. Yeast-2-hybrid results indicated that *Pb* G protein α subunits do not interact with the ScGpr1 C-terminal cytoplasmic domain (Table 3.15-1).

Table 3.15-1. Number of positive clones after screening pGAD- *ScGPR1*$^{679\text{-}961}$-RM-Lib.

	pGBK-*PbGPA1*	pGBK-*PbGPA2*	pGBK-*PbGPA3*	pGBK-*ScGPA2*
pGAD-*ScGPR1*$^{679\text{-}961}$-RM-Lib	0	0	0	50

A positive control was made by screening pGAD-*ScGPR1*$^{679\text{-}961}$-RM-Lib with pGBK-*ScGPA2* (for construction see Table 3.11). As expected, a number of positive clones were obtained, indicating that most mutations of *ScGPR1* disrupt its protein structure, preventing its interaction with ScGpa2. Although *Pb* G protein α subunits can not interact with ScGpr1, we still can not exclude the possibility that *Pb* has a *GPR* gene(s) that interacts with G proteins. Recently, we have cloned a putative G protein-coupled receptor which is 47% identical to the G protein-coupled receptor GprF from *A. nidulans* (data not shown; Han et al, 2004). It needs further

examination to determine its relationship with G proteins in *Pb*.

As we have 4 segments of *PbCYR1* cloned into both bait and prey vector, a yeast-2-hybrid analysis was made to test intra-protein interactions of adenylate cyclase or interactions generated from its homo-polymers. Most members of Class III adenylate cyclases, with a single copy catalytic domain in their genes, exist as homodimers formed by interactions of identical catalytic domains (Linder and Schultz, 2003). However, there is no evidence to prove the case for fungal adenylate cyclases. Our pilot yeast-2-hybrid analysis with *Pb* adenylate cyclase could not find any interactions between the existing functional domains (Table 3.15-2).

Table 3.15-2. Yeast-2-hybrid analysis for interactions between PbCyr1 domains.

	pGBK-*PbCYR1*$^{1-678}$	pGBK-*PbCYR1*$^{600-1316}$	pGBK-*PbCYR1*$^{1302-1876}$	pGBK-*PbCYR1*$^{1648-2100}$
pGAD-*PbCYR1*$^{1-678}$	–	–	–	–
pGAD-*PbCYR1*$^{600-1316}$	Not tested	–	–	–
pGAD-*PbCYR1*$^{1301-1876}$	Not tested	Not tested	–	–
pGAD-*PbCYR1*$^{1347-2100}$	Not tested	Not tested	Not tested	–

However, we could not exclude the possibility of some false negatives because some protein interactions may not show in the artificial yeast-2-hybrid system. For example, *PbCYR1*$^{1-678}$ in prey vector pGADT7 was not functional in yeast-2-hybrid system (data not shown). The reason for this is not clear. It is noteworthy that the catalytic domain *PbCYR1*$^{1648-2100}$, when cloned into both bait and prey vectors, did not yield positives in the yeast-2-hybrid system, suggesting that homodimers of *Pb* adenylate cyclase do not form. However, it is still too early to make a firm conclusion.

3.16 *Pb* G Protein γ Subunit PbGpg1 Interacts with Truncated Adenylate Cyclase PbCyr1$^{1-678}$ and Truncated β Subunit PbGpb1 but not Full-Length β Subunit PbGpb1 and Full-Length α Subunits PbGpa1-3

A G protein γ subunit gene *PbGPG1* was cloned by using degenerate PCR and SSP-PCR with pDNR-*Pb01*cDNA Library plasmids (Dr. G. Chen, unpublished data). The gene encodes a protein with 91 amino acids and a molecular weight of 10 kDa (data not shown). PbGpg1 is highly conserved, with 62% identity to the G protein γ subunit Gng1 of *N. crassa* and 22% identity to Ste18 of *S. cerevisiae.* When analyzed by SMART (EMBL), PbGpg1 contains a GGL (G protein gamma subunit-like) motif, i.e. an extended α-helical polypeptide that is supposed to form a stable dimer with the G protein β subunit but not to contact with the α subunits.

The full-length *PbGPG1* was cloned into bait vector pGBKT7 and prey vector pGADT7 respectively (a gift from Dr G. Chen). Yeast-2-hybrid analysis was done (by Dr G. Chen) and the results are shown in Table 3.16-1 as below.

Table 3.16-1. Yeast-2-hybrid: PbGpg1 interacts with PbCyr1$^{1-678}$ but not full-length PbGpb1 and full-length PbGpa1-3.

	Constructions for PbCyr1, PbGpb1 and pbGpa1-3 (see Table 3.11)				
Constructions for PbGpg1	pGBK-*PbCYR1*$^{1-678}$	pGBK-*PbGPB1*	pGBK-*PbGPA1-3*	pGAD-*PbGPB1*	pGAD-*PbGPA1-3*
pGAD-*PbGPG1*	+	-	-	Not applicable	Not applicable
pGBK-*PbGPG1*	Not applicable	Not applicable	Not applicable	-	-

The results indicated that PbGpg1 interacts with PbCyr1$^{1-678}$ but not full-length PbGpb1 and full-length PbGpa1-3. In order to further test the interaction between PbGpg1 and PbGpb1, truncated *PbGPB1* constructions were made by deleting one or more WD40 domain(s) and cloning into prey vector pGADT7 (the positions of

WD40 domains are illustrated in Figure 3.8-2). This job was finished by Dr G. Chen and illustrated in Table 3.16-2 as below.

Table 3.16-2. Constructions with truncated *PbGPB1* cloned into pGADT7.

Constructions	Descriptions
pGAD-*PbGPB1*WD1	WD40 domains 2-7 were deleted.
pGAD-*PbGPB1*WD1-2	WD40 domains 3-7 were deleted.
pGAD-*PbGPB1*WD1-3	WD40 domains 4-7 were deleted.
pGAD-*PbGPB1*WD1-4	WD40 domains 5-7 were deleted.
pGAD-*PbGPB1*WD1-5	WD40 domains 6-7 were deleted.
pGAD-*PbGPB1*WD1-6	WD40 domain 7 was deleted.
pGAD-*PbGPB1*WD2-7	WD40 domain 1 and coiled-coil domain were deleted.
pGAD-*PbGPB1*WD6-7	Coiled-coil domain and WD40 domains 1-5 were deleted.

These truncated *PbGPB1* constructions were then used to test their interactions with pGBK-*PbGPG1* in the yeast-2-hybrid system (this job was done by Dr G. Chen). The results are displayed in Table 3.16-3 as below.

Table 3.16-3. PbGpg1 interacts with truncated PbGpb1.

	pGAD-*PbGPB1* WD1	pGAD-*PbGPB1* WD1-2	pGAD-*PbGPB1* WD1-3	pGAD-*PbGPB1* WD1-4	pGAD-*PbGPB1* WD1-5	pGAD-*PbGPB1* WD1-6	pGAD-*PbGPB1* WD2-7	pGAD-*PbGPB1* WD6-7
pGBK-*PbGPG1*	-	+	+	+	+	-	-	-

The results indicated that *PbGPG1* can interact with pGAD-*PbGPB1*WD1-2, pGAD-*PbGPB1*WD1-3, pGAD-*PbGPB1*WD1-4 and pGAD-*PbGPB1*WD1-5 but not with pGAD-*PbGPB1*WD1, pGAD-*PbGPB1*WD1-6, pGAD-*PbGPB1*WD2-7 and pGAD-*PbGPB1*WD6-7, indicating that the *Pb* G protein γ subunit PbGpg1 interacts with the

truncated β subunit PbGpb1. The shortest truncated form of PbGpb1 that interacted with PbGpg1 was the coiled-coil domain plus the first and the second WD40 domain.

3.17 Truncated PbGpb1 Interacts with Full-Length PbGpa1 but not Full-Length PbGpa2 and PbGpa3.

Full-length PbGpb1 can not interact with full-length PbGpa1-3 as indicated in the following Table 3.17-1.

Table 3.17-1. Yeast-2-hybrid: full-length PbGpb1 do not interact with full-length PbGpa1-3 (see Table 3.11 for the constructions).

	pGBK-*PbGPA1*	pGBK-*PbGPA2*	pGBK-*PbGPA3*	pGBK-*PbGPB1*
pGAD-*PbGPA1*	Not tested	Not tested	Not tested	-
pGAD-*PbGPA2*	Not tested	Not tested	Not tested	-
pGAD-*PbGPA3*	Not tested	Not tested	Not tested	-
pGAD-*PbGPB1*	-	-	-	Not tested

Random mutagenesis libraries were used in screening to search for such potential interactions. However, no positive clones were found. The results are illustrated as below in Table 3.17-2.

Table 3.17-2. Yeast-2-hybrid: RM libraries screening for potential interaction between PbGpb1 and PbGpa1-3.

RM Library	**pGBK-*PbGPA1***	**pGBK-*PbGPA2***	**pGBK-*PbGPA3***	**pGBK-*PbGPB1***
pGAD-*PbGPA1*-RM-Lib	Not tested	Not tested	Not tested	0
pGAD-*PbGPA2*-RM-Lib	Not tested	Not tested	Not tested	0
pGAD-*PbGPA3*-RM-Lib	Not tested	Not tested	Not tested	0
pGAD-*PbGPB1*-RM-Lib	0	0	0	Not tested

To further test the interaction between PbGpb1 and PbGpa1-3, the truncated

PbGPB1 constructions listed in Table 3.16-2 were used to co-transform with pGBK-*PbGPA1-3* (see Table 3.11) in yeast-2-hybrid system. The results are shown below in Table 3.17-3 (finished by Dr G. Chen).

Table 3.17-3. Yeast-2-hybrid: Truncated PbGpb1 interacts with full-length PbGpa1 but not full-length PbGpa2 and 3.

	pGAD-*PbGPB1* WD1	pGAD-*PbGPB1* WD1-2	pGAD-*PbGPB1* WD1-3	pGAD-*PbGPB1* WD1-4	pGAD-*PbGPB1* WD1-5	pGAD-*PbGPB1* WD1-6	pGAD-*PbGPB1* WD2-7	pGAD-*PbGPB1* WD6-7
pGBK-*PbGPA1*	-	+	+	+	-	-	-	-
pGBK-*PbGPA2*	-	-	-	-	-	-	-	-
pGBK-*PbGPA3*	-	-	-	-	-	-	-	-

The results indicate that truncated G protein β subunit PbGpb1 interacts with full-length α subunit PbGpa1 but not full-length PbGpa2 and 3. The shortest form of PbGpb1 for interaction with PbGpa1 is the coiled-coil domain plus the first and the second WD40 domains. This is the same as for PbGpg1.

Chapter VI

Discussion

4.1 An Effective Way of Cloning Genes from *Pb*

Before the genome era, most yeast genes were cloned by cross hybridization on the basis of homologous gene sequences from other organisms. For example, *ScGPA1* and *ScGPA2* were cloned by using a rat Gα gene (Nakafuku et al, 1987 and 1988); fission yeast *gpa2* gene was cloned by using the Gα genes from rat and *Dictyostelium discoideum* (Obara et al, 1991; Isshiki et al, 1992). The adenylate cyclase gene from fission yeast was cloned using low-stringency hybridization with the budding yeast adenylate cyclase gene (Young et al, 1989); *Candida*'s adenylate cyclase gene *CaCYR1* was cloned by degenerate PCR (Mallet et al, 2000). In our experience, degenerate PCR is an effective way to clone *Pb* genes but attention should be paid to avoid primers spanning introns. In fact, intorns are probably prevalent in *Pb* genes as all the genes we have cloned indicate the existence of introns, for example, *PbGPA2* even contains 9 introns. However, introns are not a major problem in *S. cerevisiae*, as 70% of yeast genes are intron free including those in the cAMP signaling pathway. Therefore, cloning genes from *Pb* is more difficult than from *S. cerevisiae*. This may explain why by 2000, relatively few genes had been cloned from *Pb*. To date, we still need to clone genes one by one for an organism if its genome information is not complete. In order to overcome the trouble caused by introns, a cDNA library of good quality is needed. Amplification of genes with degenerate primers from a *Pb* cDNA library is much more direct and convenient than from genomic DNA. However, for some genes with low abundance of transcription or no expression in a specific biological state or a stage of development, there could be a problem when a cDNA library is used. In this case, genomic DNA or its library

is necessary.

Our cloning was done by degenerate PCR using a combination of cDNA and genomic DNA libraries. This proved to be effective as all three isoforms of G protein α subunits have been cloned. In addition, only one *TPK* gene was amplified from *Pb* when different combinations of very degenerated PCR primers were used. However, when PbTpk1 was used in searching homologous sequences in a *Pb* EST database, additional *TPK* polypeptide sequences could be found. Further investigation is needed to ensure if they are true isoforms or simply strain variation. Interestingly, *A. nidulans*, *N. crassa* and *M. grisiea*, which are phylogenetically related to *Pb*, each have 2 PKA catalytic subunits, falling into 2 groups during evolution (Figure 3.9-4). PbTpk1 belongs to one group but another potential PKA catalytic subunit from *Pb* seems missing in the other group. We are confident to obtain this missing gene with further degenerate PCR if it does exist in *Pb*. With RACE PCR or single specific PCR, full length genes of major components of the cAMP signaling pathway have been cloned. In fact, our cDNA library is of quite good quality because it is markedly representative of expressed genes in *Pb*. We cloned most components of cAMP signaling pathway from it, even for the Gγ protein which is quite small with a length of only 0.3 kb.

Recently, several EST (expressed sequence tag) projects have been finished by scientists in Brazil (Goldman et al, 2003; Felipe et al, 2003 and 2005). However, all these sequence tags only represent about 25-80% of the whole transcriptome. In fact, most of our genes are not included in their findings, indicating the limitation of their cDNA libraries. Therefore, a project for whole genome sequencing is also necessary.

Given the existence of intron sequences in most of the genes and differential splicing in some of the genes, there will be difficulties in analyzing the genomic data. We strongly suggest that both genomic and cDNA libraries be used in combination or in complementation with each other in cloning genes from *Pb*.

4.2 Clues from the Primary Structure of the Major Components of the cAMP Signaling Pathway

Including the work presented in this thesis, we have cloned the central components of the cAMP-signalling pathway of *Paracoccidioides brasiliensis* using the genomic and cDNA libraries. Phylogentic analyses of PbCyr1, PbGpa1,2,3, PbGpb1, PbGpg1, PbRas1, PbTpk1 and PbTpk11 indicate that *Pb* is evolutionally related to *Aspergillus nidulans* and *Penicillium marneffei.* However, the biological behaviors of these fungi are diverse including geographical distributions. *A. nidulans* is only a weak opportunistic fungal pathogen. *P. marneffei* is an opportunistic fungal pathogen in Southeast Asia, while *Pb* is restricted to South America. This will bring difficulty in judging the exact function of a protein just by analyzing the homology of its primary structure with a protein having a known function. A detailed description of a *Pb* protein's function will depend on analysis in a tractable genetic system of *Pb* which at present does not exist. However, that does not mean we can not do anything without this system. We can still obtain some basic information from cloning genes and analyzing primary structures. Gene expression and biochemical analyses will also be useful in analyzing a protein's function(s).

The *Pb* adenylate cyclase PbCyr1 is not a membrane protein but it could be a membrane-associated protein similar to ScCyr1. Like other fungal adenylate

cyclases, PbCyr1 has 4 domains: an RA domain, LRR domains, a PP2Cc domain and a CYCc domain. It has little homology with mammalian adenylate cyclase except in the catalytic CYCc domain (Fig. 3.3-5). These domains can be effectively expressed in the yeast *S. cerevisiae* and *E. coli* (data not shown). The N-terminal region (1-678; with RA domain included) of PbCyr1 can interact with G proteins, Ras1 and actin in yeast two-hybrid systems, while constructs with other domains do not interact with any proteins tested. There are 3 possibilities for this. First, these proteins are non-interactive. Secondly, the yeast two-hybrid system is an artificial system and sometimes false negatives occur without known reasons. Thirdly, the segmented gene construction might be unstable and degraded in the yeast cells. However, this seems unlikely because the PP2Cc domain and CYCc domain were easily expressed in *E. coli* (data not shown).

There are 3 different transcripts for *Pb* adenylate cyclase that differ in their splicing at the first intron to produce 2 types of truncations, PbCyr1$^{\Delta 58}$ and PbCyr1$^{\Delta 58\text{-}64}$. This is not a surprise because differential splicings can also be found in an adenylate cyclase of the malaria parasite *Plasmodium falciparum* (Muhia et al, 2003). However, this region of truncation does not overlap with the conserved residues responsible for interaction with Gpa2 in *S. pombe* (Ivy and Hoffman, 2005). The behavior of N-terminal PbCyr1$^{\Delta 58\text{-}64}$ is the same as that of N-terminal full-length PbCyr1 in terms of interacting with G proteins, Ras1 and actin (data not shown). The function of this truncation still needs further examination.

The conserved motif aXXaXXX (a represents a hydrophobic amino acid and X represent any amino acid) or LXXXXXX at the C-terminal end (1916-1930) of the

yeast *S. cerevisiae* adenylate cyclase is also conserved in PbCyr1 (1953-1967). This motif repeat is responsible for the coiled-coil domain that interacts with the cyclase-associated protein CAP in yeast (Nishida et al, 1998), suggesting the existence of a CAP homologue in *Pb*.

Adenylate cyclases can be classified into 3 groups (Linder and Schultz, 2003). Class I cyclases are present in many gram-negative bacteria such as *E. coli*. Class II cyclases serves as extracellular toxins in certain pathogens such as *Bacillus anthracis* or *Bordetella pertussis*. Class III adenylate cyclases are phylogenetically related multi-domain proteins. They are found in prokaryotes and in eukaryotes, including bacteria, metazoans, protazoans, mammals and fungi. There are 6 conserved amino acids in the catalytic domains of the Class III adenylate cyclases, serving 3 principle functions in catalysis: two aspartate residues for metal-cofactors (Mg^{2+} or Mn^{2+}) binding; a lysine and aspartate pair for selecting ATP as substrate, and an arginine and asparagine pair for transition-state stabilization (Linda and Schultz, 2003). The current understanding of catalytic domains of mammalian adenylate cyclase indicates that they form homo- or heterodimers for function. The catalytic activity of mammalian adenylate cyclases is activated or inhibited by the binding of Gαs or Gαi proteins to the C2 and C1 cytoplasmic domains, respectively, to increase or decrease the interactions of these domains (Dessauer et al, 2002). Yeast *S. cerevisiae* adenylate cyclase is always purified as a complex with molecular weight from 670 to 890 kDa (Wang et al, 1992), indicating that it is a dimer. Adenylate cyclases from both *Pb* and *S. cerevisiae* contain all the 6 conserved amino acids for catalytic functions. Given that the primary structure of *Pb* adenylate cyclase is similar to that of *S. cerevisiae*, PbCyr1 should also exist as a dimer complex. It is interesting that

the catalytic domain alone of *S. cerevisiae* adenylate cyclase is active (Kataoka et al, 1985), indicating that dimer formation is independent of other domains within adenylate cyclase itself. In contrast to mammalian cells, G proteins do not act on the catalytic domain of adenylate cyclase in *Pb*. Nor is there evidence that there is an interaction between the two catalytic domains themselves (Table 3.15-2). The question is how the dimer is formed. Interestingly, CAP forms a dimer using its last 27 amino acid residues (Freeman et al, 1996). Its N-terminus interacts with the C-terminal end of adenylate cyclase (Nishida et al, 1988). In addition, CAP was co-purified with adenylate cyclase (Wang et al, 1992). Therefore, dimer formation of adenylate cyclase could be brought by the cyclase associated protein CAP.

Gene duplication is an important mechanism for acquiring new biological functions during evolution. Many genes are present in multiple isoforms in fungi. For example, in the cAMP signaling pathway of the yeast *S. cerevisiae* there are 2 G protein α subunits, 2 Ras proteins and 3 cAMP-dependent protein kinases. These proteins, although homologous in their primary sequences, have divergent functions. In *Pb*, 3 isoforms of G protein α subunits have been cloned (this thesis). We believe that they have evolved from duplication of a single copy gene as the introns are localized in the same positions in the genes (Figure 3.7-4). Yeast *S. cerevisiae* G protein α subunits function divergently, one controls cAMP levels for pseudohyphal growth and one controls mating through the MAPK pathway (Borges-Walmsley and Walmsley, 2000; Hoffman, 2005a). Therefore, the 3 Gα proteins in *Pb* may have different functions. The data in this thesis indicate that they all act on adenylate cyclase but supposedly have different roles in regulating adenylate cyclase. They might also work on additional pathways such as the MAPK pathway, components of

which have been identified in an EST analysis (Felipe et al, 2005).

In *S. cerevisiae*, there are two artificial dominant active alleles $GPA2^{G132V}$ and $GPA2^{Q300L}$, and a dominant negative allele $GPA2^{G299A}$ (Harashima and Heitman, 2005). These amino acids are conserved in *Pb* Gα subunits for potential point mutations to yield dominant active or negative alleles. The corresponding mutations for PbGpa1 are G42V, Q204L and G203A; for PbGpa2 are G46V, Q210L and G209A; for Gpa3 are G45V, Q208L and G207A. In addition, the GXG^{16}XXG motif is also conserved in PbRas1. Therefore, $PbRAS1^{G16V}$ should be a dominant active allele. It is worth testing the biological behaviors of these dominant active or negative alleles once the transformation system for *Pb* has been established.

All Gα proteins have N-terminal lipid modifications, i.e. myristoylation and palmitoylation, for membrane targeting (Cabrera-Vera et al, 2003). The site of myristoylation is the glycine at the second position in the consensus sequence MGXXXS. Palmitoylation occurs on cysteine(s) near the N-terminus. All the *Pb* Gα proteins isolated here retain these consensus sequences. They are MGCGMS for PbGpa1, MGCASS for PbGpa2 and MGGCCS for PbGpa3. Myristoylation of Gα proteins is responsible for membrane localization and coupling to the membrane receptor but does not affect the interaction with Gβγ (Song et al, 1996). Therefore, the 3 Gα proteins from *Pb* are likely to be myristoylated, palmitoylated and membrane targeted. In addition, PbRas1 and PbGpg1 both have the conserved CAAX (A for aliphatic amino acids) motif (Kuroda et al, 1993), suggesting that they are both palmitoylated, farnesylated and targeted to the membrane. These findings suggest that all the G proteins of *Pb* are membrane anchored and probably coupled to

membrane receptors. When using *S. cerevisiae* Gpr1 to search a *Pb* EST database, however, no homologous sequence can be found. Recently, we have cloned a putative G protein-coupled receptor from *Pb* (data not shown). It is 47% identical to the G protein-coupled receptor GprF from *A. nidulans* (Han et al, 2004). Its relationship with G proteins need to be tested.

In *S. cerevisiae*, there are 3 catalytic subunits of cAMP-dependent protein kinases, which have probably acquired different functions during evolution by development of specific domains for different protein interactions. Tpk2 is responsible for both pseudohyphal and invasive growth, while Tpk1 and Tpk3 play negative roles (Robertson and Fink, 1998; Pan and Heitman, 1999). The 3 Tpk proteins are highly conserved at their C-terminal but are highly divergent at their N-terminal ends. The distinct N-terminal extension of Tpk2 is glutamine-rich (29%) compared with Tpk1 (9% glutamine) and Tpk3 (3% glutamine). One of the two catalytic subunits of PKA in *C. albicans* is also glutamine-rich (19% for CaTpk2) in its distinct N-terminus, while the other is not (5% for CaTpk1). Both Tpk proteins of *C. albicans* are involved in regulation of dimorphism (Bockmuhl et al, 2001). We have cloned one copy of a *TPK* gene in *Pb*, namely *PbTPK1*. The PbTpk1 is also glutamine-rich (15%) in its N-terminus. Although the function of the poly-glutamine stretch is still unknown, it is possible that PbTpk1 belongs to a class of fungal PKA catalytic subunits that evolved to specifically regulate dimorphism.

In *S. cerevisiae*, Tpk1 and Tpk3 are specialized to inhibit filamentous differentiation. Epistasis analyses suggest that Tpk1 and Tpk3 exert their negative effects through element(s) upstream of PKA itself (Pan and Heitman, 1999). In fact, there is a

cAMP accumulation in cells growing on glucose-containing media when the Tpk proteins are disrupted (Nikawa, et al, 1987). This indicates that Tpk1 and Tpk3 function in a negative feedback loop that represses cAMP production. The target of this feedback has not been found but it may include adenylate cyclase. In *Pb*, although we have cloned only one catalytic subunit (*PbTPK1*) of PKA, we cloned another Tpk-like gene *PbTPKL1*. A similar situation also prevails in *S. cerevisiae* and *C. albicans*. In *S. cerevisiae*, the Tpk-like protein, Sch9, functions in parallel to both the Gpr1-Gpa2-cAMP pathway and the MAPK pathway. The Sch9 kinase is required for normal vegetative growth and heat shock sensitivity when Gpa2 is activated (Lorenz et al, 2000; Xue et al, 1998). However, further experiments are required to test if the PbTpk-like protein is in the cAMP pathway or not. Interestingly, the PbTpk-like gene transcript level correlated well with peaks for PbCyr1, suggesting that it plays an important role in controlling the morphological switch (Chen et al, 2006a, manuscript for publication). In this thesis, the relationships between adenylate cyclase and the heterotrimeric G proteins, a Ras protein and actin have been delineated by yeast two-hybrid assays. Further experiments indicated that PbTpk1 also interacts with the N-terminus of adenylate cyclase (data not shown). The relationships between adenylate cyclase and PbTpkl1 have not been tested. It will be interesting to see if a negative feedback loop could exist via either Tpk1 or Tpk-like protein in the cAMP pathway of *Pb*.

We have cloned a *PbTUPA* gene which is homologous to the *S. cerevisiae TUP1* gene. The *TUP1* gene plays a major role in glucose repression in yeast (Williams and Trumbly, 1990). In addition, it can regulate other phenotypes such as yeast floculence, sporulation and mating (Williams and Trumbly, 1990). When yeast cells

are grown on glucose, enzymes that are needed for utilizing alternate carbon sources are turned off, i.e. repressed. When glucose is exhausted, these enzymes are derepressed. The *TUP1* gene is probably at the end of a signaling pathway that is responsive to glucose. Glucose is one of the stimulants that are involved in triggering the cAMP signaling pathway in yeast. Therefore, we suspect that *TUP1* is an element in the cAMP pathway or an element that interacts with the pathway, but currently there is no genetic evidence to support this. Interestingly, PbTupA contains seven WD40 repeats which are homologous to the *Pb* G protein β subunit PbGpb1 (13% identity), suggesting a similar function to the Gβ subunit. Indeed the structure of the *S. cerevisiae* Tup1 contains a seven-bladed β propeller which can be super-imposed over the similar structure of Gβ proteins (Sprague et al, 2000). It could be involved in protein-protein interactions such as binding to transcriptional factors or binding to an element in a signaling pathway. Alternatively, it could bind to DNA directly to exert its repressive function. The function of PbTupA needs further examination.

4.3 Yeast Two-Hybrid Assay

Genetic analysis is an essential way to delineate a signaling transduction pathway but a detailed map depends on the solid evidence of protein-protein interactions, which are usually demonstrated by yeast two-hybrid assays and co-immunoprecipitation. However, in most cases for cell signaling pathways, protein interactions are not constitutive but exhibited only in a transient state. In the yeast *S. cerevisiae*, for example, Rgs2 physically interacts with Gpa2 but this interaction is very weak in yeast two-hybrid analyses and in immunoprecipitation assays; a strong interaction is only observed when Gpa2 is in the GTP-binding state (Versele et al, 1999). The

reason for this is probably that a specific and tight binding of proteins depends on the existence of a signal which triggers a change in protein conformation(s). Proteins can change between 'switched-on' and 'switched-off' states corresponding to its conformation with the ability to bind or not to its partner. This makes it very difficult to find a protein-protein interaction simply by a routine yeast two-hybrid screening. To overcome this difficulty, we devised an effective way of finding an interaction by screening a random mutagenesis library when the interaction can not be observed with the full-length wild type gene. We supposed that random mutagenesis could bring the protein, which otherwise is incompetent for binding, into a conformational state competent for binding or to remove a part of the protein that may have a negative regulatory role for binding. In this thesis, the interactions of *Pb* adenylate cyclase with PbGpa2, PbGpa3, PbRas1 and PbActin were all found in this way. This is consistent with some observations of other researchers. For example, in the yeast *S. cerevisiae*, Gpa2 is also involved in sporulation regulation through direct inhibition of a meiosis-specific protein kinase Ime2 (Donzeau and Bandlow, 1999), which was cloned by yeast two-hybrid screening with Gpa2. It is noteworthy that most of the isolated clones are a C-terminal fragment of the regulatory domain. Further analysis indicates that although Gpa2 interacts with the full-length Ime2, the strength of interaction is only 1/40 of that of the interaction with the C-terminal fragment of Ime2. It is interesting to note that this interaction is also dependent on the GTP-binding state of Gpa2. We could imagine that because Gpa2 is a yeast gene that it can easily transform into a transient GTP-binding state in yeast cells under the cognate regulator(s). In yeast two-hybrid analysis, a dominant active $Gpa2^{G132V}$ can interact with full-length Ime2 but an inactive $Gpa2^{G299A}$ can not interact with the full-length Ime2. However, both the active and inactive forms of

Gpa2 can interact well with the C-terminal fragment of Ime2 (Donzeau and Bandlow, 1999). These results indicate that the conformational change of Gpa2 triggered by GTP is responsible for its interaction with Ime2, possibly by changing the conformation of both Gpa2 and Ime2 and exposing the C-terminal interacting domain of Ime2. Once the N-terminus is deleted from Ime2, the interacting domain is readily exposed so that it interacts with both the active and inactive Gpa2 in a constitutive and strong manner. This example is highly consistent with our experience. In some cases, yeast two-hybrid analyses, especially for a transient protein interaction in a cell signaling pathway, the interactions may be too weak to detect. This is especially the case when the two proteins are not from the yeast *S. cerevisiae*; since then their conformations might not be adjustable by yeast regulators and then they may not be competent for interaction. Point mutations or deletion of a part of one or both proteins may help to expose the interacting domains and facilitate their interaction.

4.4 All the Three Gα prteins and a Gβ Protein Interact with Adenyalte Cyclase in *Paracoccidioides brasiliensis*

The cAMP signaling pathway of *Paracoccidioides brasiliensis* is very important as demonstrated by the fact that all 3 G protein α subunits, the G protein β subunit and βγ dimer can interact with adenylate cyclase PbCyr1 at its N-terminus. During the course of our study, it was reported that Gpa2 binds to the N-terminus of adenylate cyclase to cause its activation in *S. pombe* (Ogihara et al, 2004; Ivey and Hoffman, 2005). Our result is still unique in fungal biology because it is the first evidence that all the Gα proteins can directly interact with adenylate cyclase in an organism. Our study is also the first to demonstrate that Gβ and the Gβγ dimer can directly interact

with adenylate cycalse in fungi. On the other hand, we have established that PbGpa1 interacts with PbGpb1, which also interact with PbGpg1, indicative of the formation of a canonical Gpa1-Gpb1-Gpg1 heterotrimer. In contrast, Gpa2 and Gpa3 seem to work alone or with other yet unidentified partners. Analysis of gene transcripts by real-time PCR indicated that *PbCYR1*, *PbTPK1*, *PbGPA1, 2, 3* and *PbGPG1* are all expressed at higher levels in the yeast form than mycelial form (Chen et al, 2006a, manuscript for publication). This suggests that an elevated level of cAMP supports the yeast form of *Pb*. However, the addition of cAMP into the media retarded the mycelium to yeast transition, but did not prevent the yeast to mycelium transition, nor did it induce the transition of yeast at 37°C (Chen et al, 2006b, manuscript for publication). This result is in contrast to previous observation that exogenous cAMP blocked the yeast to mycelium transition (Paris and Duran, 1985).

G proteins are a family of guanine nucleotide-binding proteins that are involved in a variety of receptor-mediated signal transduction systems, including sensory, neurotransmitter and hormone signalling (Lambright et al, 1996). For example, Gs, and Gi are involved in hormonal stimulation and inhibition. The best-understood pathways of G protein action are in mammalian cell systems. These G proteins, consisting of α, β, and γ subunits, exist as inactive heterotrimers with GDP bound to the α subunit and are coupled to a cell surface receptor (Hoffman, 2005a). Receptor stimulation leads to a conformational change in Gα and exchange of GDP for GTP on the G protein α subunit, activating Gα. The activated Gα then dissociates from the βγ subunits, which do not undergo a conformational change. Both α and βγ subunits are then able to modulate diverse intracellular effectors, including adenylate cyclase, cyclic GMP phosphodiesterase, and phospholipase A2. Hydrolysis of GTP to GDP

turns off the Gα, which then reassociates with free βγ to return to the inactive Gαβγ state. The duration of G protein signalling depends on the intrinsic GTPase rate of the Gα subunit but is also modulated by extrinsic factors such as the Gβγ dimer and RGS proteins (regulators of G protein signalling; Hoffman, 2005a).

In mammalian cells, an excess of the Gβγ dimer promotes reassociation with Gα, inhibiting Gα stimulated adenylate cyclase activity. In addition, Gβγ alone can activate or inhibit adenylate cyclase by direct interaction (Diel et al, 2006). The working mechanism of the canonical heterotrimeric G proteins in *S. cerevisiae* is probably similar to that of the mammalian ones but with differences. In the yeast pheromone pathway, it is the Gβγ dimer that activates the pathway. The Gα subunit turns off the signal by association with the Gβγ dimer. The G protein β subunit Ste4 is probably the limiting component of the active Gβγ dimer because the activated pheromone response generated by overexpression of Ste4 (β) was abolished by deletion of Ste18 (γ) but overexpression of Ste18 alone does not induce the pheromone response (Cole et al, 1990). In addition, overexpression of Gpa1(α) neutralizes the biological effect of overexpression of the β subunit Ste4 (Cole et al, 1990). Deletion of *GPA1* leads to the same phenotype as the overexpression of the β subunit Ste4, i.e. haploid-specific cell cycle arrest, indicating that Gβγ is constitutively active without damping by the Gα subunit (Cole et al, 1990; Whiteway et al, 1990). These findings suggest that a proper stoichiometry in the levels of the G-protein subunits exists in a normal mating pheromone response. The ratio of G protein α, β, and γ subunits probably determine the state of the cells in response to pheromone. In fact, various phenotypes exist in response to different concentrations of pheromone. It was supposed that the pheromone pathway is capable of existing in

a state not fully active or inactive (Cole et al, 1990). Within the cells, the concentration of Ste4 (β) is probably lower than that of Ste18 (γ). Overexpression of Ste4 further potentiates the pheromone response, while overexpression of Gpa1 sequesters the Gβγ dimer into an inactive Gαβγ trimer, attenuating the pheromone response. In addition, activated Gpa1 is capable of producing an adaptive response by interacting with its own regulator or effector. For example, Gpa1 can directly interact with an upstream RGS (regulators of G-protein signalling) Sst2 that probably stimulates conversion of Gpa1 to the GDP-bound state and promotes recovery after pheromone-induced growth arrest (Dohlman et al, 1995; Dohlman et al, 1996). Furthermore, the GTP-charged Gpa1 directly interacts with activated Fus3, a downstream element of the MAPK pathway, performing an adaptive-negative control by inhibiting localization of Fus3 in nuclei (Metodiev et al, 2002; Blackwell et al, 2003). Recently, it has also been found that the GTPase-deficient $GPA1^{Q323L}$ allele can also activate the pheromone pathway by directly interacting with an RNA binding protein Scp160 (Guo et al, 2003). The possible position of Scp160 in the pathway is downstream of the receptor and upstream of Gβγ. Therefore, either Gα or Gβγ alone can activate the pheromone MAPK pathway and the traditional model should be modified.

A similar situation has been noted in *S. pombe*, where the Gβ Git5 is transcribed at much lower levels than Gpa2 and Git11 (Hoffman, 2005a). It has been hypothesized that the uncomplexed Gpa2 could be associated with adenylate cyclase to form an inactive complex: glucose activation of the signaling pathway would lead to the Gpa2-GTP complex, released from the Git5-Git11 dimer, being swapped for Gpa2-GDP bound to adenylate cyclase that would then become activated (Hoffman,

2005a).

In *P. brasiliensis*, there is one canonical heterotrimeric G proteins, consisting of PbGpa1(α), PbGpb1(β) and PbGpg1(γ). The latter two subunits form a tight Gβγ dimer supposedly but there is also an opportunity that PbGpb1 works alone when transcript levels of PbGpb1 and PbGpg1 are imbalanced (Chen et al, unpublished data). Both the PbGpa1 and the Gβ (or Gβγ dimer) modulate the same effector, i.e. adenylate cyclase. The working mechanism is probably analogous to that in the pheromone pathway of *S. cerevisiae*. For example, PbGpb1-PbGpg1 could activate the adenylate cyclase while PbGpa1 could negatively regulate adenylate cyclase, and meanwhile sequester the Gβγ dimer and inactivate it. Alternatively, PbGpa1 may play the active role while the *Pb* Gβγ dimer may play the negative role by interacting with both adenylate cyclase and Gpa1. This situation is similar to that of Gpa2-Gpb1/2 interaction in *S. cerevisiae* if both Gpa2 and Gpb1/2 directly act on the same target, adenylate cyclase. Therefore, either PbGpa1 or PbGpb1 activates the pathway, while the opposite component plays a negative role by directly damping its partner and exerting an adaptive negative effect via adenylate cyclase. It is also possible that both Gpa1 and the PbGpb1-PbGpg1 dimer activate adenylate cyclise, while one sequesters another by direct interaction.

The ratio of PbGpa1 and the Gβγ dimer (or simply PbGpb1) could also be crucial in determining the phenotype of the *Pb* cells. It is possible that overexpression of one component may cause the inability of the other to be fully sequestered, leading to a phenotype dominated by the overexpressed component. In fact, similar to the graded phenotype in yeast mating, the temperature induced

morphological transition in *Pb* is a long progress, taking 3 weeks for complete transition (da Silva et al, 1999). This indicates that the transition is a complex set of events, including multiple genes that could be turned on or off, up-regulated or down-regulated, at the transcription level. One possible event is a change in the balance of the G protein subunits. According to our results, the α subunit PbGpa1 and the β subunit PbGpb1 probably require conformational changes for interaction. Similarly, full-length PbGpb1 may not be in the required conformation to interact with PbGpg1, possibly due to the C-terminus blocking the interaction. The formation of the PbGpb1-PbGpg1 dimer may require Hsp30 as a chaperone since we found a high propensity to clone this gene from a yeast two-hybrid library screened with full-length PbGpb1 (Chen et al, unpublished results). This is not surprising as recent studies have revealed a role for phosducins as chaperones for the Gβγ complex (Lukov et al, 2005). PbGpa1 and PbGpb1 may be loosely associated and either of them alone may be capable of modulating the adenylate cyclase. Indeed, our gene transcriptional analysis with real-time PCR indicated that there was an increasing imbalance in the *PbGPA1* and *PbGPB1* transcript levels during the process of morphological transition (Chen et al, unpublished data). In mycelium the transcript levels for *PbGPA1*, *PbGPB1* and *PbGPG1* are nearly equivalent, suggesting that the signal is dependent upon GTP-induced dissociation of the Gαβγ complex. However, during the mycelium to yeast transition, the *PbGPB1* transcript levels were 11 fold higher than *PbGPA1* after 24 hours, but towards the end of the transition, after 240 hours, the *PbGPA1* transcript levels were 3 times higher than those for *PbGPB1*. In contrast, the *PbGPG1* transcript levels were either equivalent to, or less than, those for *PbGPA1*. These results lead us to suggest that during the transition, PbGpb1

would be in excess and freely available to modulate the activity of adenylate cyclase and govern the morphological switch. Indeed, the addition of exogenous cAMP, which retards the morphological switch, suppresses the *PbGPA1/PbGPB1* imbalance but stimulates *PbCYR1* transcription, albeit with a maximum after 72 hours instead of 24 hours (Chen et al, unpublished data).

It is interesting that all the interacting proteins including G proteins, PbRas1 and PbActin work on adenylate cyclase through its N-terminal one third which is over 800 amino acids residues away from the catalytic domain. This is in contrast to the situation in mammalian cells, in which G proteins modulate the activity of adenylate cyclase by binding to the catalytic domain (Tesmer et al, 1997). In *Pb*, the signals from regulators such as G proteins, PbRas1 and PbActin have to be relayed to the catalytic domains. In our study, we did not detect any interaction between RA, LRR, PP2Cc and CYCc domains of adenylate cyclase, but these might be only be induced by the binding of effectors, such as Gpa1 to the N-terminal domain.

Yeast Ras2 is a direct activator of adenylate cyclase and mediates the glucose signalling. Ras2 associates with adenylate cyclase via an interaction with the RA domain (Kido et al, 2002). Ras2 has a second binding site at the C-terminal end of adenylate cyclise in the presence of CAP (Shima et al, 2000), indicating that the RA domain and the C-terminus are in proximity in the 3-D structure. Interestingly, a mutant adnenylate cyclase $Cyr1^{K1876M}$ allele abolishes glucose- and acidification-induced increase in cAMP but still maintains the basal level of cAMP (Vanhalewyn et al, 1999). This mutation probably leads to a conformation change of the catalytic domain which in turn fails to communicate with the activated N-terminal domains.

The K1876 is conserved in *Pb*; consequently, it will be interesting to see how it affects *Pb* adenylate cyclase activity and further more how it affects the communication between the catalytic domain and the N-terminal domains.

In *S. cerevisiae*, there is one canonical heterotrimeric G protein, consisting of Gpa1(α), Ste4(β) and Ste18(γ). The Gα can directly interact with Gβγ as indicated by a yeast two-hybrid analysis (Ongay-Larios et al, 2000). A mutation of Ste4 with Leu-132 in the second WD40 domain replaced by phenylalanine leads to a defective interaction between Gα and Gβγ, indicating that the second WD40 domain (but it is the first blade) is essential for the interaction. Crystal structures of mammalian heterotrimeric G proteins revealed that Gα changes its conformation when dissociated from the Gβγ but that Gβ does not change its conformation following dissociation (Lambright et al, 1996). The Gβ folds into a symmetric propeller structure containing seven blades of β sheets. For each blade, there are 4 antiparallel β sheets radiating outwards from a central axis. The first 3 β sheets of an individual blade come from the C-terminus of a WD40 sequence but the fourth β sheet comes from the N-terminus of the next WD40. Therefore, the N-terminus of the first WD40 and the C-terminus of the last WD40 form the 7^{th} blade. The N-terminal region of Gβ is involved in the coiled-coil interaction with the N-terminus of Gγ. The Gβγ use its N-terminal coiled-coil domain to interact with its downstream effector (Ongay-Larios et al, 2000).

In contrast to the situation in *S. cerevisiae*, PbGpa1 can not directly interact with PbGpb1 in the yeast two-hybrid system. There are 3 possible explanations for this. Firstly, it might be that PbGpa1 can directly interact with PbGpb1 but this is not

apparent in the yeast two-hybrid system. This situation is similar to the Gpa2-Krh1 interaction in *S. cerevisiae*, where Gpa2 can not interact with full-length Krh1 (kelch repeat homologue; was named Gpb2 by Harashima and Heitman, 2002) but interacts with the C-terminus of Krh1 (Batlle et al, 2003). Secondly, in mammalian cells, the G protein β and γ subunit are treated as a single unit because they are tightly associated throughout purification (Neer and Clapham, 1988). However, PbGpa1 could not interact with a PbGpb1-linker-PbGpg1 fusion protein (Chen et al, unpublished data); it is notable that neither PbGpa1 nor PbGpg1 interacted with the full-length PbGpb1, which needed to be truncated at the C-terminus to enable the interaction (Table 3.16-1 and 3; Table 3.17-1 and 3). Thirdly, full-length PbGpb1 can interact with Hsp30 which is an integral membrane protein that might serve as a chaperone, like phosducins, for assistance in Gβγ dimer assembly (Lucov et al, 2005; Chen et al, unpublished dara). It is interesting that the Gγ PbGpg1 and the Gα PbGpa1 bind to the same first two WD domains on PbGpb1. It is not clear if they compete for binding to PbGpb1. In *S. cerevisiae*, a HSP70 chaperone Ssa1 directly interacts with Cdc25 and positively controls the production of cAMP (Geymonet et al, 1998). Deletion of Ssa1, for example, leads to a decrease in Cdc25 content in the cells but not at the transcript level. In *Pb*, Hsp70, which has been proposed to be a cellular thermometer, is highly correlated with the dimorphic switch (da Silva et al, 1999). It usually binds to and stabilizes unfolded proteins after heat shock. Hsp30 may be another important chaperone in *Pb*. Its function may be different form Hsp70 because its expression is down-regulated during the mycelium to yeast transition, whilst the expression of Hsp70 is up-regulated (data not shown; da Silva et al, 1999). Given the suggestion that Hsp30 regulates the assembly of the Gβγ dimer, the down-regulation of Hsp30 may make Hsp30 unavailable for Gα and Gβ.

Without the assistance of Hsp30, Gα and Gβ would be in a conformation not suitable for direct interaction, and would thus work independently. This would give even finer control of adenylate cyclase activation. In fact, the morphological transformation is quite a slow process, taking more than 2 or 3 weeks to go from mycelia to yeast. In this case, it is not a surprise that the organism responds to temperature change so slowly because the regulation is so complicated and indirect.

Our data indicate that PbGpa2 and PbGpa3 do not interact with PbGpb1. They must either act alone or with yet unidentified proteins, possibly with kelch repeat proteins. Similar situations have been observed in budding yeast and fission yeast. In budding yeast, Gpa2 can not form a typical heterotrimeric protein with Ste4 and Ste18 but forms an atypical heterotrimeric protein with kelch repeat proteins Gpb1/Krh2 and Gpb2/Krh1 (Harashima and Heitman, 2002; Batlle et al, 2003). Similarly in fission yeast, Gpa1 in the pheromone pathway fails to form a heterotrimeric protein with the known Gβγ subunits Git5/11 (Hoffman, 2005a). The Gpa1 of fission yeast possibly associates with the kelch repeat protein Ral2 as indicated by genetic studies (Hoffman, 2005a). In *S. cerevisiae*, the kelch repeat protein Gpb2/Krh1 (or Gpb1/Krh2) works downstream of Gpa2 (Batlle et al, 2003). In addition, Both Gpb2/Krh1 and Gpa2 could directly interacts with adenylate cyclase (Harashima and Heitman, 2002; Hoffman, 2005a). However, there is no direct evidence that Gpa2 and Gpb2/Krh1 can directly interact with adenylate cyclase to date.

4.5 *Pb* RAS Protein Acts on Adenylate Cyclase

Mammalian RAS proteins can activate adenylate cyclase in the yeast *S. cerevisiae* but neither the mammalian RAS nor the yeast RAS proteins can regulate mammalian

adenylate cyclase (Kataoka et al, 1985). Even in the fission yeast *S. pombe*, there is no indication that adenylate cyclase is regulated by RAS proteins although there is an obvious RA domain (Hoffman 2005a). It seems that additional information in the primary structure of adenylate cyclase determines RAS interaction. Our data indicates that *Pb* is the second fungus whose adenylate cyclase is regulated by RAS protein. In yeast *S. cerevisiae*, Ras can bind directly to adenylate cyclase and the binding is dependent on GTP but not dependent on farnesylation and CAP (Kido et al, 2000). However, activation of adenylate cyclase depends on farnesylation of Ras and the N-terminus of CAP. Interestingly, full-length PbRas1 did not interact with adenylate cyclise but interacted with its N-terminus or C-terminus truncated forms (Fig. 3.14-3). The possibility is that PbRas1 is not in the GTP-binding state in the yeast cell in which there is no cognate regulators such as GEF. In *S. cerevisiae*, the adenylate cyclase complexes extracted from the membrane do not include Ras proteins because the molecular sizes of the complexes were not affected by the presence or absence of the RAS2 protein (Wang et al, 1992). Therefore, Ras proteins are not tightly associated with adenylate cyclise, suggesting a transient interaction.

In *S. cerevisiae*, the conserved N-terminal half of Ras appeared to be involved in adenylate cyclase activation while the variable C-terminal half is not crucial as the C-terminal half deletion mutation *ras1*Δ or *ras2*Δ behaved the same as the wild type in terms of the cAMP level produced (Hurwitz et al, 1995). In addition, deletion of the C-terminal half of Ras2 generated a constitutively active Ras2Δ protein which could activate adenylate cyclase independent of GTP, and which bypassed a GEF mutation $cdc25^{ts}$ (Marshall et al, 1987). Therefore, the C-terminal half of Ras appears to play a negative regulatory role in adenylate cyclase activation. The adenylate cyclase

binding domain of Ras has been mapped to the N-terminus and a peptide spanning residues 17-44 can completely inhibit the binding of Ras to adenylate cyclase (Ohnishi et al, 1998). This is consistent with our results because full-length PbRas1 does not interact with adenylate cyclase but the truncated version of PbRas1 (1-83) interacts with adenylate cyclase.

In *S. cerevisiae*, the RAS interaction site does not fall within the leucine-rich repeats. However, the leucine-rich repeats are indispensable for activation (Suzuki et al, 1990). The leucine-rich repeats form a special stereo structure that is essential for protein-protein interactions (Kajava, 1998). There is probably an intramolecular interaction within adenylate cyclase so that the signals caused by interaction with G proteins and Ras, in the N-terminal third of adenylate cyclase, can be relayed to the catalytic domain. Currently, we have no evidence of interdomain interactions in adenylate cyclase. Given that *Pb* adenylate cyclase could be a dimer, regulation of its activity could be much more complicated than anticipated.

4.6 *Pb* Actin Acts on Adenylate Cyclase

PbActin can interact directly with the N-terminal domain of *Pb* adenylate cyclase. This is not without precedence because recent studies have established a direct interaction of adenylate cyclase with actin in mammalian cells (Bajo et al, 2004). Previous studies have also established a connection between nutritional response signalling and cytoskeleton remodelling in yeast. In *S. cerevisiae*, adenylate cyclase is tightly associated with CAP; a bifunctional protein, with its N-terminal 168 residues being responsible for mediating farnesylated Ras activation of adenylate cyclase and its C-terminal 158 residues being responsible for mediating normal cell

morphology and reaction to nutrient deprivation and excess (Nishida et al, 1998; Vojtek et al, 1991). The latter function can be suppressed by overexpression of the profilin, an actin- and polyphosphoinositide-binding protein, indicating a possible role for CAP in cytoskeletal association. In fact, *cap-* cells bud randomly and are defective in actin distribution (Vojteck et al, 1991). The function of CAP is probably for cellular localization of adenylate cyclase to actin filaments other than the Ras-adenylate cyclase interaction. Indeed, the C-terminal half of CAP can directly bind to actin (Freeman et al, 1995). In addition, its proline-rich region binds to a SH3 containing protein Abp1, another actin-binding protein which can localize CAP and adenylate cyclase to cortical actin (Freeman et al, 1996). It is possible that the two functions of the N- and C-terminus of CAP are correlated. CAP might be responsible for a subcellular localization of adenylate cyclase. However, in our study, we established that adenylate cyclase can interact directly with actin alone. This means that CAP might be redundant for the subcellular localization of adenylate cyclase in *Pb*. There is still no evidence as to whether adenylate cyclase of the yeast *S. cerevisiae* can bind actin or not. If not, it might be CAP that acts as a scaffold to hold actin and adenylate cyclase together, while in *Pb*, adenylate cyclase can localize itself to actin without the assistance of CAP. PbCyr1 can interact with the C-terminus truncated actin but not full lengh actin (Fig. 3.13 and Fig. 3.14-3). This can be explained by 3 possibilities. Firstly, adenylate cyclase interacts with the actin polymer rather than the monomer. Secondly, adenylate cyclase interacts with actin with the aid of a third partner that is probably CAP in *Pb*. In this case, CAP might be responsible for the spatial distribution of adenylate cyclase to make it accessible for G proteins or Ras; or to concentrate it at specific developmental structures, where it could locally increase the cAMP level. Thirdly, actin could regulate a specific

conformation of adenylate cyclase, transiently activating it in response to a specific signal.

4.7 Future Work

Although the current model for the activation of heterotrimeric G protein dictates that Gα dissociates from Gβγ and reassociates with it thereafter, this is not necessarily the case in reality. It has been demonstrated in the yeast *S. cerevisiae* that a nondissociable Ste4-Gpa1 fusion protein with only one amino acid as a linker is fully active in transducing the pheromone signal (Klein et al, 2000). In another study, dissociation was not required for Gα to activate adenylate cyclase (Bar-Sinai et al, 1992). Therefore, we propose that most of the molecules involved in G protein signalling pathway likely form a large complex that could include the receptor, G proteins and effectors. Upon signalling, instead of dissociating and reassociating, these components could change their conformations to relay the signal downwards. This hypothesis is possible as all these upstream components are membrane-associated. However, it is hard to prove this because in most situations the down stream effectors of Gα and Gβγ are different. Our data indicates that in *Pb*, both the Gα and Gβγ directly work on the N-terminal end of adenylate cyclase. It would be interesting to crystallize the Gα(GDP)-adenylate cyclase dimer, Gα(GppNHp)-adenylate cyclase dimer and Gβ-adenylate cyclase dimer, respectively. Comparing these structures will tell us the relationships between these three components in both the situation when the pathway is activated and when it is inactivated.

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