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CELL SIGNALLING IN  
*PARACOCCIDIOIDES BRASILIENSIS*

by

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A thesis submitted in partial fulfillment of the requirement for

the Degree of Doctor of Philosophy

at Durham University

May 2006



29 NOV 2006

Abstract of thesis entitled  
**Cell Signalling in *Paracoccidioides brasiliensis***

submitted by

**Daliang Chen**

for the degree of Doctor of Philosophy

at Durham University

in May 2006

*Paracoccidioides brasiliensis* (*P. brasiliensis* or *Pb*) is the etiological agent of paracoccidioidomycosis 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  $\alpha$  subunits (*PbGPA1*, *PbGPA2* and *PbGPA3*), a G protein  $\beta$  subunit (*PbGPB1*), a G protein  $\gamma$  subunit (*PbGPG1*), a cAMP dependent protein kinase catalytic subunit (*PbTPK1*), a cAMP dependent 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*<sup>1-678</sup> 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*<sup>1-678</sup>, *PbCyr1*<sup>600-1316</sup>, *PbCyr1*<sup>1302-1876</sup> and *PbCyr1*<sup>1648-2100</sup>. It was demonstrated that *PbCyr1*<sup>1-678</sup>, where the Ras association domain resides, can interact with truncated versions of *PbGpa2*, *PbGpa3*, *PbRas1*, and *PbActin*, i.e., *PbGpa2*<sup>1-102</sup>, *PbGpa2*<sup>1-183</sup>, *PbGpa3*<sup>1-160</sup>, *PbGpa3*<sup>1-213</sup>, *PbRas1*<sup>1-83</sup>, *PbRas1*<sup>26-238</sup>, and *PbActin*<sup>1-314</sup>.

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  $\alpha$  subunits, G protein  $\beta$  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

## Acknowledgment

First of all, I am greatly indebted to my supervisors Dr M. Ines Borges-Walmsley and Professor Adrian R Walmsley. It is they who led me into this interesting scientific research field and provide me with a real career opportunity. The work in this thesis could not have been finished without their encouragement, instruction and supervision. I am also very grateful to their painstaking tutelage and correction on the writing of this thesis. Wealth of knowledge and working experience has been accumulated due to their kindness, helpfulness and patience.

Special thanks go to Mr. Simon Padbury, Ms. Diane Hart and Ms. Ray Cowan for their excellent technical efforts in maintaining the Category III lab at Durham University and University of Glasgow. Their job was professional, demanding they pay attention to detail.

I also give many thanks to Dr Gongyou Chen for his effort in cloning *PbGPB1* and *PbGPG1*, and producing some very nice Y2H results. His good suggestions on experiments and general encouragement are highly appreciated.

Here, I should also thank many other colleagues from the lab that assisted and encouraged me during the work leading to this thesis but I will not list their names one by one in fear of missing one of them.

Last but not the least, I am grateful to Wellcome Trust and Durham University for the provision of funds that supported my research, my career, and the life of my family.

## 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- $\beta$ -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
<i>Pb</i>	<i>Paracoccidioides brasiliensis</i>
<i>Pb01</i>	<i>Paracoccidioides brasiliensis</i> strain ATCC 90659
PCM	Paracoccidioidomycosis
PCR	Polymerase Chain Reaction
<i>pfu</i>	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  $\mu\text{m}$ ) 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*,  $\alpha$ -1,3-glucan is the main polysaccharide in the yeast cell wall, and there are only traces of  $\beta$ -1,3-glucan. In contrast,  $\beta$ -1,3-glucan is the only form of glucan in the mycelial cell wall. The predominance of  $\alpha$ -1,3-glucan in the yeast cell wall may have a role in protection against host defense because phagocytic cells do not digest  $\alpha$ -1,3-glucan (Goldman et al, 2003), while  $\beta$ -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  $\alpha$ -1,3-glucan and more  $\beta$ -1,3-glucan are avirulent (Silva et al, 1994), supporting the role of  $\alpha$ -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  $\beta$ -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  $\beta$ -galactofuranose. The mycelial membranes contain equivalent amounts of GIPCs. The yeast membranes predominantly comprise the  $\beta$ -galactofuranose-containing GIPC which was reactive with sera from patients with PCM (Levery et al, 1998). The extract of GIPC without the  $\beta$ -galactofuranose was not reactive with the patients' sera. The immunological reactivity of the  $\beta$ -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. glucanases 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 (Vicentini 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  $\beta$ -estradiol plays an important role in preventing females to get an overt infection. *In vitro* experiments confirmed that  $\beta$ -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  $\beta$ -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  $\beta$ -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 subclinical 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  $\alpha$ -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 marrow). 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 immunosuppression 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  $\gamma$ -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 (Restrepo and Moncada, 1974). Research on immunodiagnosis has been emphasized on searching for novel 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 itraconazole. 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 ketoconazole. 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 paracoccidioidomycosis (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 phosphodiesterase) 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 dibutyryl 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 racemosus* 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

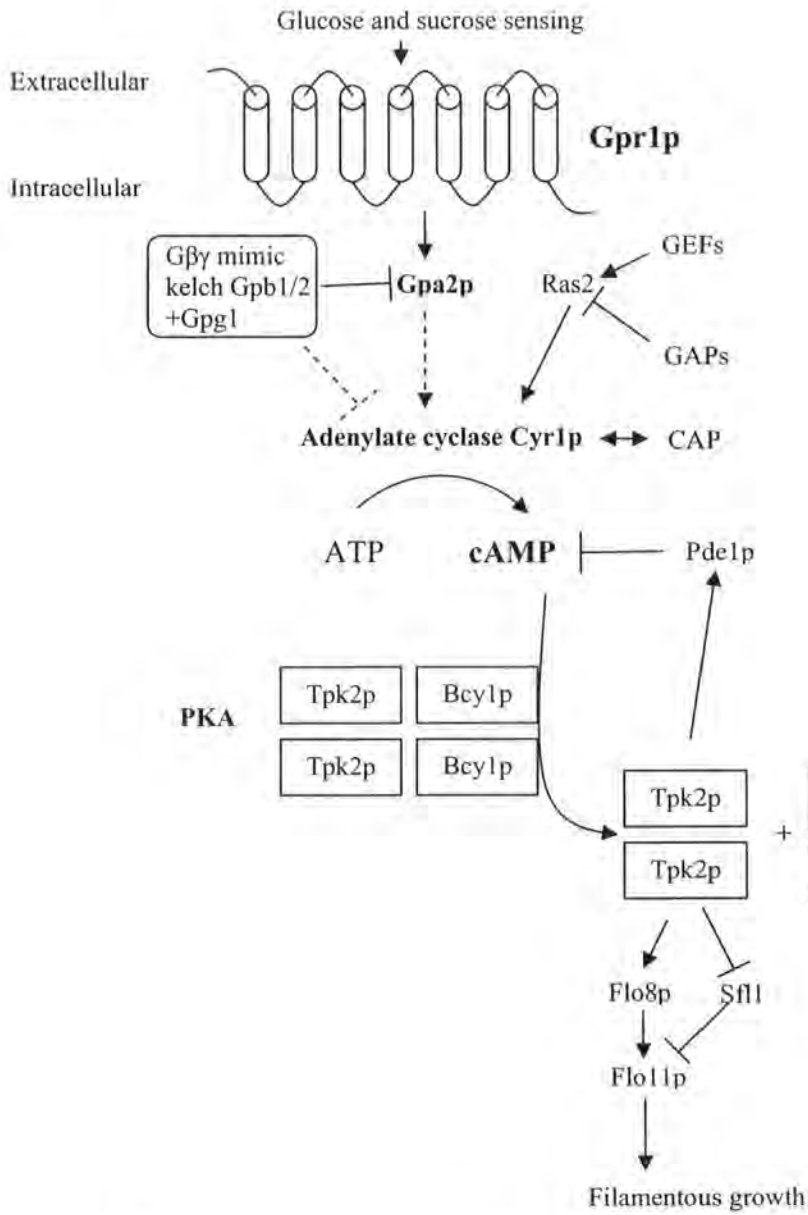
pathway (Lengeler et al, 2000). In fact, most of our knowledge of the cell signaling pathways controlling morphological switching in pathogenic fungi came from the research on pseudohyphal growth of *S. cerevisiae* (Borges-Walmsley and Walmsley, 2000). Current research indicates that pathogenic fungi 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. brasiliensis* 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* (cyclic AMP requirement), 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 adenylate cyclase activity from the membrane portion to the cytosolic portion (Mitts et al, 1990). In another experiment, the adenylate cyclase activity located predominantly in the high speed pellet fraction when adenylate cyclase containing the LRR repeats was over-expressed in *E. coli*, while the adenylate 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, adenylate cyclase activity in *ras1ras2bcy1* 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 adenylate 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 adenylate 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*<sup>G19V</sup> 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) domain 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 cyclase 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*<sup>K1876M</sup>, 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*<sup>G19V</sup> 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  $\alpha$  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*<sup>R273A</sup> 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  $\Delta gpa2$  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  $\Delta mep2/\Delta mep2$  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  $\Delta mep2/\Delta mep2$  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<sup>G19V</sup>* 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*<sup>G19V</sup> cells by overexpression of the phosphodiesterase 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 adenylate 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<sup>G19V</sup>* 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<sup>G19V</sup>* 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-bcyl* 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<sup>G19V</sup>*) produced GTP-dependent (GTP-independent for *RAS2<sup>G19V</sup>*) 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. cerevisiae* 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 activates 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<sup>G19V</sup>* stimulates pseudohyphal 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<sup>G19V</sup>* 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  $\alpha$  subunit. *GPA2* is homologous to mammalian G protein  $\alpha$  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  $\Delta gpa2/\Delta gpa2$  mutant strain has a defect in pseudohyphal growth that can be rescued by exogenous cAMP in the media (a  $\Delta ras2/\Delta ras2$  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  $\Delta gpa2$  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  $\Delta gpa2/\Delta gpa2$  mutation. None of the deletions of the MAPK pathway components *STE20*, *STE11*, *STE7* and *STE12* blocks the dominant active  $GPA2^{G132V}$  to stimulate 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*<sup>G132V</sup> 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  $\alpha$ ,  $\beta$  and  $\gamma$  subunits. Signaling through the conformational change in the G protein-coupled receptor promotes GDP-GTP exchange on the G protein  $\alpha$  subunit, and releases the G $\beta\gamma$  complex. In mammalian cells, both G $\alpha$  and G $\beta\gamma$  can induce downstream signaling events (Hoffman, 2005a). However, the yeast G $\alpha$  protein Gpa2 lacks the traditional  $\beta\gamma$  subunits (Lorenz and Heitman, 1997). The pheromone-responsive  $\beta\gamma$  complex (Ste4 and Ste18) is not involved in pseudohyphal growth and thus has no relationship with Gpa2. There are another 8 candidate  $\beta$  genes and 3 candidate  $\gamma$  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 G $\beta$  subunit mimics, and Gpg1 which serves as G $\gamma$  mimic (Harashima and Heitman, 2002; Battle et al, 2003; Harashima and Heitman, 2005). The G $\beta\gamma$  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  $\beta$  subunits (Ito et al, 1994). Gpg1 is a small protein containing only 126 amino acid residues. It is similar in size to known  $G\gamma$  subunits and its secondary structure is predicted to be two linked  $\alpha$ -helical domains, similar to known  $G\gamma$  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  $G\beta\gamma$  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<sup>R237A</sup> (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  $\Delta gpb1\Delta gpb2$  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  $\Delta gpa2$  to the  $\Delta gpb1\Delta gpb2$  mutant only partially reduces the hyperfilamentous growth. Furthermore, the expression of Flo11 in  $\Delta gpa2\Delta gpb1\Delta gpb2$  cell is two- to three-fold lower than that in  $\Delta gpb1\Delta gpb2$  cells, but higher than in  $\Delta gpa2$  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  $\beta$  propeller structure (Adams et al, 2000). Therefore, the G $\beta\gamma$  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  $\Delta gpb1\Delta gpb2$  strain. However, the transient peak of cAMP production upon glucose stimulation exists in either single  $\Delta gpb1$  or single  $\Delta gpb2$  mutants, but diminishes in a double  $\Delta gpb1\Delta gpb2$  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 G $\beta$  subunits which promote interactions of the G $\alpha$ -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 phospholipase C, which hydrolyzes the membrane phospholipids phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) 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 filamentation MAPK pathway) only caused a 2-fold decrease. Perhaps Plc1 is downstream of Ras2 because dominant active *RAS2*<sup>G19V</sup> does not rescue the defect caused by  $\Delta$ *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<sup>R237A</sup>* allele (supposed to induce decreased intrinsic GTPase activity) in a double *Δgpr1Δras2* mutant (Xue et al, 1998). Expression of another dominant active *GPA2<sup>G132V</sup>* 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  $\Delta tpk2/\Delta tpk2$  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  $\Delta bcy1$  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  $\Delta gpa2/\Delta gpa2$  or  $\Delta gpr1/\Delta gpr1$  double deletion (Pan and Heitman, 1999). The  $\Delta tpk2/\Delta tpk2$  completely suppresses filamentation caused by the dominant active allele *GPA2*<sup>G132V</sup>. On the other hand, the  $\Delta tpk2/\Delta tpk2$  can only partially suppress filamentation caused by dominant active *RAS2*<sup>G19V</sup>, 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  $\Delta ras1 \Delta ras2 \Delta pde2 \Delta ste12$  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 adenylyate cyclase which catalyzes the formation of cAMP. Adenylyate cyclase is tightly bound to cyclase-associated protein CAP, which is essential for Ras activation and might play a role in subcellular localization of adenylyate cyclase (Shima et al, 1997; Zelicof et al, 1996). Both guanine nucleotide-binding proteins Gpa2 and Ras activate adenylyate 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 adenylyate cyclase activation (Toda et al, 1985). The RAS proteins directly act on adenylyate cyclase to maintain the basal cAMP level (Borges-Walmsley and Walmsley, 2000; Kido et al, 2002). The activation of adenylyate 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 G $\alpha$  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 Gy mimic Gpg1 form a G $\beta\gamma$  mimic which interacts with Gpa2 and negatively regulates it. The G $\beta\gamma$  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 MAT $\alpha$  and MAT $a$ . When MAT $\alpha$  and MAT $a$  cells are co-cultured, they conjugate and form a third cell type, the diploid MAT $\alpha$ /MAT $a$  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  $\alpha$ -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* ( $\alpha$  subunit), *STE4* ( $\beta$  subunit) and *STE18* ( $\gamma$  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 G $\alpha$  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  $\alpha$  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  $\alpha\beta\gamma$  heterotrimers. Genetic data indicated that oligopeptide mating pheromones, a-factor and  $\alpha$ -factor, bind to Ste3 and Ste2 respectively, and activate the heterotrimeric G proteins. Both Gpa1 and Ste4 ( $\beta$ ) 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  $\alpha$  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  $\beta$  and  $\gamma$  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 G $\beta\gamma$  dimer contributes to the interaction between the G $\alpha$  subunit and the receptor by directly interacting with Gpa1 and causing a conformational change in this G $\alpha$  subunit or by directly contacting the receptor itself (Blumer and Thorner, 1990). Indeed, the G $\beta$  Ste4 can directly interact with both the pheromone receptor and the G $\alpha$  Gpa1 in yeast two-hybrid analyses (Ongay-Larios et al, 2000). A G $\beta$  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  $G\alpha$  protein is upstream of  $G\beta\gamma$  in mediating the signal (Ongay-Larios et al, 2000). Although the receptor coupling of  $Gpa1$  requires  $G\beta\gamma$ , the association of  $Gpa1$  with the membrane is independent of the  $\beta$  and  $\gamma$  subunits (Blumer and Thorner, 1990).

Upon pheromone stimulation, the  $G\alpha$   $Gpa1$  becomes GTP charged, uncoupling it from the receptor, and concertedly, releases the  $G\beta\gamma$  dimer. In yeast, it is the  $G\beta\gamma$  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 overexpression of *STE4* ( $\beta$ ) in the absence of *STE18* ( $\gamma$ ) did not produce the pheromone response (Cole et al, 1990; Whiteway et al, 1990). These results suggest that the  $G\beta\gamma$  dimer stimulates the pheromone signaling pathway, while the  $G\alpha$   $Gpa1$  plays a negative regulatory role. The function of *Ste18* is to aid proper folding of  $G\beta$  *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  $G\beta\gamma$  is the conserved mitogen-activated protein kinase (MAPK) cascade, which includes *Ste11*, *Ste7*, and *Fus3* (Lengeler et al, 2000). Free  $G\beta\gamma$

(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 G $\beta\gamma$  recruits Ste5 to the membrane, bringing Ste5 in proximity to the cell surface-associated Ste20 (Pryciak and Huntress, 1998). The interaction between G $\beta\gamma$  and Ste5 is constitutive and does not depend on pheromone activation, while the interaction between G $\beta\gamma$  and Ste20 depends on pheromone stimulation. Therefore, G $\beta\gamma$  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 G $\beta\gamma$  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<sup>G19V</sup>* 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).

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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 filamentation 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 filamentation 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  $\Delta tpk2/\Delta tpk2$  mutant strains loses the unipolar budding pattern and agar invasion but retains the ability for cell elongation, while  $\Delta ste12\Delta ste12$  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<sup>G19V</sup> 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  $\Delta ste12$  strain and overexpression of Ste12 in a  $\Delta flo8$  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  $\Delta ste12$  strain with Flo8 overexpression, the cells were shorter but had a denser network. In the  $\Delta flo8$  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<sup>G19V</sup> or Cdc42<sup>I2V</sup>. 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 G $\beta\gamma$  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 G $\alpha$  (Git8) and G $\beta\gamma$  (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 G $\beta$  Git5 and abolished by a dominant active Gpa2 (Hoffman, 2005b). The G $\gamma$  Git11 was obtained from a two-hybrid screening with G $\beta$  Git5 (Landry and Hoffman, 2001). Gpa2 functions downstream of GPCR and G $\beta\gamma$  because the defect of *fbp1* transcriptional repression caused by  $\Delta git3$ ,  $\Delta git5$ , or  $\Delta git11$  is suppressed by overexpression of the wild type *gpa2* or an activated *gpa2*<sup>R176H</sup> allele. Deletion of G $\beta\gamma$  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 G $\beta\gamma$  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 G $\alpha$  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-Walmsley 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), CaBcyl (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 *CaRASI<sup>G19V</sup>* allele enhances hyphal growth (Feng et al, 1999; Maidan et al, 2005a). The defect caused by the *Caras1Δ* 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 *Caras1Δ* 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

telisporos (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 G $\alpha$  protein Gpa3 (Regenfelder et al, 1997; Kruger et al, 1998), the G $\beta$  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 result in

disease, indicating that filamentation and virulence are divergently controlled (Mayorga and Gold, 1998). There are 4 G $\alpha$  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 $\Delta$  can be rescued by exogenous cAMP, indicating that *gpa3* is upstream of *uac1* (Kruger et al, 1998). Recently, the G $\beta$  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 $\Delta$  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 $\Delta$  strain, the *bpp1 $\Delta$  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, MAT $\alpha$  and MAT $\alpha$ . 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. MAT $\alpha$  cells can also filament and sporulate in response to nitrogen starvation in the absence of MAT $\alpha$  cells or in response to pheromone secreted by MAT $\alpha$  cells (Lengeler et al, 2000). This perhaps makes MAT $\alpha$  more prevalent in the environment and clinical isolates. In addition, MAT $\alpha$  cells are more virulent than MAT $\alpha$  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<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> (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), G $\alpha$  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 *GPAL* (Xue et al, 2006).  $\Delta$ *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 *GPAL* 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 *GPAL* 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 G $\beta$  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.**

<u>Strains</u>	<u>Source</u>	<u>Application</u>
<i>Pb01 (P. brasiliensis)</i>	90659 from ATCC	DNA and RNA extraction
<i>E. coli</i> NovaBlue	Novagen	Competent cell for blue/white selection
<i>E. coli</i> XL1-Blue MRF'	Stratagene	Host of $\lambda$ ZAP phage
<i>E. coli</i> XL10-Gold	Stratagene	Ultra-competent cell
<i>E. coli</i> SOLR	Stratagene	Phagemid excision
<i>E. coli</i> DH5 $\alpha$	Invitrogen	Competent cell for cloning
<i>E. coli</i> BL21 (DE3)	Novagen	Protein expression
<i>E. coli</i> BL21 (DE3) pLYS	Novagen	Protein expression
<i>E. coli</i> Rosetta 2 (DE3)	Novagen	Protein expression
<i>E. coli</i> Rosetta 2 (DE3) pLYS	Novagen	Protein expression
<i>S. cerevisiae</i> AD1-8'	From Prof A Goffean	DNA extraction
<i>S. cerevisiae</i> AH109	Clontech	Yeast two hybrid

**Table 2.2-2 Culture media used in this thesis.**

<u>Media</u>	<u>Composition of media</u>	<u>Application</u>
LB	10 g/L NaCl, 5 g/L yeast extract, 10 g/L bactotryptone	<i>E. coli</i> 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	<i>Pb01</i> culture

**Table 2.2-3 Vectors used in this thesis.**

<u>Vectors</u>	<u>Source</u>	<u>Application</u>
pGEM-T Easy	Promega	TA cloning
pGEM-TE( <i>KpnI</i> )	pGEM-T Easy introduced with a <i>KpnI</i> site	Cloning of full length <i>PbCYR1</i>
pBSK	Invitrogen	Cloning
pYES6/CT	Invitrogen	Cloning and expression
pDNR-LIB	Clontech	<i>Pb01</i> 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 Electrophoresis**

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/ $\mu$ l. 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)
<i>PbCYR1</i>	
PbAC-F1	GTBTT <u>CACYGAYATYAAGA</u> ACWSBAC
PbAC-F2	AGAACTGCACGTCGCTTTGG
PbAC-F3	GGAAGCTGAAGGGGCTTGAG
PbAC-F4	AGTTGCGGATGGTGGGCAAATC
PbAC-F5	CCGTCCTCGAGGTGCAATGGAAACCCCATCCTCG
PbAC-F6	TATGGCAGATAGCCTTGGGCGAAAC
PbAC-F7	CAATCTGAAGAGTCTCGTTGATCTTGATATC
PbAC-F8	GTGCCCTGAAAATTTCCGGAGAGATTGGTCTTC
PbAC-F9	GTCTGGCGCCGTTGGACTGTTGCTTTACGTTTGG
PbAC-F10	GCCTCAACGCTCCACGGGCTGAGATTGAAGAGG
PbAC-F11	CCGTTGCCTATGACTTGATGTC
PbAC-F12	CCAGACACTCGTCGTCCTCAAAGAGTA
PbAC-F14	GAGAATTCCGCAATCCCTTTGAACCCTCCAG
PbAC-F15	CGTTTACGTTTGCGGGATCTCTGAAGCAAC
PbAC-F16	GAATATCGGCCGGATGAATCACATCCACTC
PbAC-F17	AGCGGCCAGAACTTAGATTTGCGGCAGCA
PbAC-F18	CAGCATGGCAAGGAGACAGCGGGAGAAAGA
PbAC-F19	GAGATGCACCTGTGTATCCAGTTCAGGAGA
PbAC-F20	CCCCAACCCGGGATTTGAGCTACTCACAGA or TCCACGTCGCTTTGGGAGACGTATCC
PbAC-F21	TTGGGATACCGTCTGCTCTTCCTCTCGCCA
PbAC-F22	AGAGAGTCCCGTGCCTGACACAGCCTTGA
PbAC-F23	TTGTCACCGCGAGTTAAAGTCCGGAGTTGA
PbAC-F24	CCACGTCGACTTGACGCGGGCGCAGTCTCA
PbAC-F25	<u>GTCATATCTCGAGCTGCAACTGAAACCCCA</u>
PbAC-F26(KpnI)	<u>GGTACCAAAATGTCTAGGAGACAGCGGGAGAAAGAT</u> AGG

PbAC-F27	AGATTTCGGATCAGGATGACAGAAGGCTGA
PbAC-F28	GATTTTCGGATCAGGATGACAGACTCGTC
PbAC-F40	CCGCATTTCCGCAGTTGCGGATGGTGGGCAAATC
PbAC-GSP1	TGATGCTGTTGAATCGTGCGTCTATCTC
PbAC-R1	TTVACCATRGGRCCSWRGATRTCCAT
PbAC-R2	CAAGTAATCCATTTCGACCAG
PbAC-R3	CCGTCAACTCGTTATAGGACAAG
PbAC-R4	NNNNCTCGAGTGGCGGCCGCTCTAGAACTA
PbAC-R5	NNNNCTCGAGGTCTATAATATCTAATAGTGATG
PbAC-R6	CAGGATTCTCAAGCCCCTTCAGCTTC
PbAC-R7	AGTCACCCATAACCGTTCAGTGTCGTAAGGCGTC
PbAC-R8	TTCCAGTGTCGTAAGGCGTCCAATATCCGACAACTC
PbAC-R9	ACTCACCTCCACCCGCGTAACCTGATGTTTC
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	CAAAGCTGGTTGTCGGTACGTCTGTTG
PbAC-R18	CAGAGGAGAGAACTTGGCATTTCGAACCA
PbAC-R19	TATCTGAGCCAACTGTCGACGGAAGAGATC
PbAC-R20	TCCAAAGCTGGTTGTCGGTACGTCTGTTGG
PbAC-R21	TGGGGATACATCGGGGGAATGTGGCCTAC
PbAC-R22	GAGTCACGCTCGCCATCTGTCATGGAACCA
PbAC-R23	CTCTTTGAGGACGACGAGTGTATTTTGAAC
PbAC-R24	AAGAAACCCCAAGCAGCATGAAGCAGGCA
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	CAAATCCACCCATATGAGCCAACGTGTCGAC
PbAC-R330	CATACTGTGGCCTGCAAGCAGGAGATTCCA
PbAC-R34(NotI)	<u>GCGGCCGCGCCGTGCTCGAAGAACTAGAACCAC</u>
PbAC-R35	CTCCATAGTTTGATATATCCTTTGATTGAC
PbAC-R36	CCATAGTTTGATATATCCTTCAGAAGACTTCCG
PbAC-R37	CATTCATAAAGCAGCTGTTCTGTGGCAGG

PbAC-GADF1(NcoI)	GGTACCACCATGGCAAGGAGACAGCGGGAGAAAG
PbAC-GADF2(NcoI)	TCGTCCATGGAAGATGAGCTGAATAACTAC
PbAC-GADR1(BamHI)	TGATTTGGATCCCGTTGATTCCCAAGTCAGC
PbAC-GADR2(BamHI)	CCCCAGGATCCTAAGATGTTTCAAAGAGTTG
PbAC-EF1(NdeI)	GGGATTCATATGGTTAATAGCACAGATCTG
PbAC-EF2(NdeI)	AGTATCCATATGGGACTGTCTCCATTAAGT
PbAC-ER1(XhoI)	CCATGGCTCGAGCGCCGTGCTCGAAGAAGT
PbAC-ER2(XhoI)	CTCGCGCTCGAGATTGTGATTAAGATAGTC
<b>PbGPA1</b>	
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	ATCGTGGCTCACGCATTTTCGAG
PbGPA1-ExGPAF1	CATATGGGTTGTGGAATGAGC
PbGPA-F1	CGGGATCCGTCATATGGGTTGTGGAATGAGCACC
PbGPA1-F1	TGTCCTTCGCTCCCGTGTTAAGACCACAGG
PbGPA1-F2	TTGCGACTATATCCTCAACCGCTTCGTCTCGCTC
PbGPA1-F3	GACTATATCCTCAACCGCTTCGTCTCGCTCAACC
PbGPA1-F4	GATTGATCGGTTTAAGGAGAACTGCCTGTTAGC
PbGPA1-F5	CTCTGAGTACGACCAGCTCCTGTTTGAAGACG
PbGPA1-F6	GACATGAAGCAGAACCCTGTACATTCCGATATCGTC
PbGPA1-F7	GTGTCATTCATCCATCTTGCTTTACGCC
PbGPA1-F8	GGCTATGGAAGGACTAGATATCCCGTTGGA
PbGPA1-F9	CTGCATGGTTTCCTTCTCAG
PbGPA1-F10	GATCAAAATGCTCTTGCTTGGTGCTGGAGA
PbGPA1-F11	GAATGACTCTGCACGATACTATTTTACTCCA
PbGPA1-R1	CGGGATCCTCATATCAGTCCACAGAGGCGAAG
PbGPA1-R2	TCCAACGGGATATCTAGTCCTTCCATAGCC or GCTTAGCCTAAACTCTCCATAGTCATCGTGTCC
PbGPA1-R3	GAGCATTTTGATCTCGTTTCTCTGCAGC
PbGPA1-R4	TCCTTGTCCTCGGTGCTCATTCCACACC
PbGPA1-R5	CCTGTGGTCTTAACACGGGAGCGAAGGACA
PbGPA1-R6	TGAAGACTGGCTGGGTTTTGCGGGAAGGA
PbGPA1-R7	CAATTACCCATGGCTTATTAGG
PbGPA1-R8	CAGTGTATTCGGCACCCCTTCGTAATCAG
PbGPA1-R9	TGTCCTGGATGATTATATCGTTGACAGCCA
PbGPA1-pYER3	GCCTAGGTCATATCAGTCCACAGAGGCGAAG
PbGPAm-F1	GCTTTTGCTTGGTGCTGTAGAGTCTGGCAAATCCAC
PbGPAm-R1	GTGGATTTGCCAGACTCTACAGCACCAAGCAAAGC

<b><i>PbGPA2</i></b>	
PbGPA2-F1	ACGAACGATACCAAACCGCGCGGTTCATCTACTC
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	TTGAGTTTAAAAGACAAAGCCACTAGCCA
PbGPA2-F10(BD-NdeI)	ATGGCCATGGAGGCCGAACATATGGGTTGC
PbGPA2-R1	TGAAGCATGTCTTGATTGTTGGGTAACCACC
PbGPA2-R2	ATCGAGGAGAATCTTGAATGCAACAACCATG
PbGPA2-R3	GCCGTCGACCACGCGTGCCCTATAGTGA
PbGPA2-R4	CATACGGATCATCTTGTTCGATCGCAGACCA
PbGPA2-R5	TGGTGCAGAAACCGAGCGTGGCACAAGAGC
PbGPA2-R6	CTGTTGGCACCTACAGAATCAGGTTGTTGA
PbGPA2-R7	CAATTCCTATCAGAACTCAATCAGCATGCA
PbGPA2-R8	AACGTAGCTCGAGTCCTTCCCCTCTGTTCA
PbGPA2-R9	CTTCGCACTTTTCAAATGCCACGGGACATG
PbGPA2-R10	GGCTTCATGCATTTGATTTGCATTTTGATCC or GAGCACAACCTCTCAAGTGAGTGCAACACCA
<b><i>PbGPA3</i></b>	
PbGPA3-F1	TCTTGGATTGCGCCAAGGACCTGATAGGAG
PbGPA3-F2	CCTAATGAAGCCGATGTGTTGCGCGCTAGA
PbGPA3-F3	TATCCTCAGTATGTATAGGAAAAGCACACC
PbGPA3-F4	AACCGTGCCGCCAAGTACTTGCTCTGGAGA
PbGPA3-F5	GTATCAGATACCTACCCACCCACAACGT or TGGTATCAGATAGGATGGGTGGGTGTTGCA
PbGPA3-F6	GTGGGTGTTGCAGTTCTGCTTCTGGGGAGA
PbGPA3-F7	CCACTTGGCCTTTTTTTCCTTTTCCTTTTCC
PbGPA3-F8	CAGCTCTGGTCCACTTGGCC
PbGPA3-F8(EcoRI)	ATCAGAATTCATGGGTGGGTGTTGCAGTTC
PbGPA3-F9	ATGCAAGATCCTACTGCTTGGTTCGGGTGA
PbGPA3-F10	TGGATTCAGCACCTTACTTCTTCGAGGAAGC
PbGPA3-F11(BD-NdeI)	ATGGCCATGGAGGCCGAACATATGGGTTGGG
PbGPA3-R1	TTCCTCCGCTGCCCCGCCAACATCGAAC
PbGPA3-R2	ACGCACTGAATACTCAATTGGCCATTGTG
PbGPA3-R3	GCGTATTCGGGTCCGCGTCAATCTGGTATG
PbGPA3-R4	CCTCTGCTCTGCATTCTCCCCAGAAGCAGA
PbGPA3-R5	CCCTTTCACAAAATACCAGAATCTTTCAGG

PbGPA3-R6	AAAGCAGTGAGTGGCCTGGGGAGTTATACG
PbGPA3-R7	GATTTGATAAGTAAGCGGCCCGATGCGGGA
PbGPA3-R8	GTCGGTGGCTTGAGTAAGGTGAGGATAAAG
<b><i>PbGPB1</i></b>	
PbGPB-F1	GCNAARATHAYGCNATGCAYTGG
PbGPB-F2	TGGGTNATGACNTGYGCNTAYGC
PbGPB-R1	GCRTANGCRCANGTCATNACCC
PbGPB-R2	CCABWKCATRCANGTCATRNCNC
<b><i>PbRAS1</i></b>	
PbRAS-F1(NdeI)	CCGCGCTTCATATGCAGCTTTGTCTAAACC
PbRAS-R1(EcoRI)	GTGATACTGTAGAATTCCACGAAACCCTC
<b><i>PbTPK1</i></b>	
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	CTGAGACGGGTCTATGTTTCGATGG
PbTPK-F40	GCTGTTTAGTTTACTGAGGAAATCGCAGCGCTTC
PbTPK-R1	ACBTMTTTKGCRAABCCRAARTC
PbTPK-R2	ATSARMAYMCCNARVSWCCACCARTC
PbTPK-R3	CTTCAAATGTCCATATCGATC
PbTPK-R4	CTAAAAGTAGATTCTCTGGCTTTAGGTCTCTG
PbTPK-R5	GTGATGGTCATGTAGATATTCCAACGCCAACG
PbTPK-R6	CTAAAAGTAGATTCTCTGGCTTTAGGTCTCGG
PbTPK1-R7	TACCTGGAACGTGCCCATAGCG
PbTPK1-R8	TAGGAATGGATGTTTAACCCGCTGCAGC
PbTPK-R9	TAATGTTCTTAATACTGGCGACATTTGGGAGTG
PbTPK1-R10	ATGTCGCGATTGAACCAGGTGTACTIONCGC
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	GANGCNTTYYTNGGNCAYGTMNG
TPKL-DR1	SWCCARAARTCNACCATYTTNGT
<b><i>PbACTIN</i></b>	
PbActin-F3	ATGCTCCTCGAGCTGTCTTCCCTTCGATCG
PbActinGADF1	CATATGGAGGAAGAAGTTGCTGCCCTCG
PbActinGADF2	GTGATTCATATGGAGGAAGA
PbActinGADR1	TTAGAAGCACTTGCGATGGACAATAGAGG
PbActin-R3	GGTACATGGTTGTGCCACCGGACATAACGA
PbACTIN-R3(EcoRI)	CGAGGGCGGTGAATTCCTATTGCATACGATC
<b><i>PbTUPA</i></b>	
cPbTUPA-F1	ACTTCTTACCTTAGGCCAGCCTCGCCCTCA
cPbTUPA-F2	ACACCCGATGGACGCTGGGTTATGAGTGGA
cPbTUPA-F3	ATCGTGCGGTACAGTTCTGGGATCCAGCGA
cPbTUPA-F4	CCCGTTCAACCCAGCCTACCAACTACCGCA
cPbTUPA-R1	ATTATATGATGACGGCGGTGATAACGAGGA
cPbTUPA-R2	ATAGCTGTAAATCCCATGTGCGAGAGTTAGG
cPbTUPA-R3	CCTTCCTCTGGCAAAGTTACGTGTTGCGCA
cPbTUPA-R7	CTGATCATCTCCATCTCCTGGAGTTGGCCA
PbTUPA-F1	MGSACYTTTGARGGNCA YAAR
PbTUPA-R1	CCARATTCKNGCNCKCATRTC
PbTUPA-R2	GTCGCTGGATCCCAGAACTGTACGCCACGA
PbTUPA-R3	CACTCATAACCCAGCGTCCATCGGGTGTC A
PbTUPA-R4	GGATATCCCAGACCTGTAGGTGGA
PbTUPA-R5	GGCAACTTGTCAGGATCCAGGTC
PbTUPA-R6	GAGTGCGGATTT CAGCCTCG
<b><i>ScGPR1</i></b>	
ScGPR1-F1	CGGGATCCGAAGTGTGACGAATAAAGC
ScGPR1-F2	GCTGGGACATCAGACTTTCTCTTGCC
ScGPR1-F3	CGGGATCCATATGATAACTGAGGGATTCCCCCG
ScGPR1-F4	CCGGATCCGGTGAAAGTAAAAGAATFAAAGCGC
ScGPR1-F5	CCGGATCCAGGAAAAACCTTGGA ACTATTCATG
ScGPR1-R1	CGGGATCCATTTTCAAACATCGCGATAC

ScGPR1-R2	GTTACTAGTTATGTCGCTGTTATCG
ScGPR1-R3	CCGGATCCTTAGATTCTTTTGAATTTGTGCC
<b>ScGPA2</b>	
ScGPA2-F1	CCGGATCCTGGGTCTCTGCGCATCTTCA
ScGPA2-F2	GGGTCTCTGCGCATCTTGAG
ScGPA2-F3	CCGGATCCGTATGGGTCTCTGCGCATCTTCA
ScGPA2-F4	ATCATGGGTCTCTGCGCATC
ScGPA2-R1	CCGGATCCGCTGTGCATTCATTGTAACAC
ScGPA2-R2	AACGCGAGAAGAGGCATGCAG
<b>Universal primers</b>	
3'AD LD Screening	AAGTGAACCTTGCGGGTTTTTCAGTATCTACGA
5'AD LD Screening	CTATTCGATGATGAAGATACCCACCAAACCCA
3'AD sequencing	AGATGGTGCACGATGCACAG
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
AP11	GTAATACGACTCACTATAGGGCACGC
AP21	TCACTATAGGGCACGCGTGGTCGAC
AP3	TACGACTCACTATAGGGCACGCGTGGTCGA
AP4	GTAATACGACTCACTATAGGGCACGCGTGG
3'DNA-BD Screening	TGGCTGCAAGCGCGCAAAAACCCCTCAAGAC
5'DNA-BD Screening	TCATCGGAAGAGAGTAGTAACAAAGGTCAAAGA
3'BD sequencing	CGCAAAAACCCCTCAAGAC
CDS/3'BamHI(2)	ATTCTAGACGCGGATCCGCGGACATGTTTTTTTTTTTT TTTTTTTTTTTTTTTTTVX
CDS/3'BamHI(3)	GATTCTAGACCGGATCCGCGGACATGTTTTTTTTTTTT TTTTTTTTTTTTTTTTTVN
CDSIII/3'BamHI	AACGCGGATCCGCGTTTTTTTTTTTTTTTTTTTTTTTT TTTTT
Creator-F1	CGACGGTACCGGACATATGCCCGGGAATC
Creator-R1	GCGCGCAAACGAATGGTCTAGAAAGCTTC
CYC1 Reverse (pYES6)	GGAGGGCGTGAATGTAAGCGTGAC
GADF1	GAAGAGAAAGGTCGAATTGGGTACCG
GADR1	CGCGGATCCGTATCGATGCCACCCGGGTGGAATC
GADR2	CGCGGATCCGTGTATCGATGCCACCCGGGTGGAATC
GAL1 Forward (pYES6)	CCTCTATACTTTAACGTCAAGGAG
5'LONG	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCA ACGCAGAGT
3'LONG	CTAATACGACTCACTATAGGGCATTCTAGAGGCCGA GGCGGCCGA
M13F1	ACCGCCTTACGCGTGTAACGACGGCCAG
M13 forward	GCGTGTAACGACGGCCAG

M13R1	CCAGGATCTCCTAGGGAAACAGCTATGACC
M13 reverse	GGGAAACAGCTATGACCATG
3-2PB	AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTVN
5-2PB	AAGCAGTGGTATCAACGCAGAGTACGCGGG
pGEMTE-SP6	AGGTGACACTATAGAATACTCAAG
PolyT	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
pQE-F1	TGTGAGCGGATAACAATTCACACAG
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 $\mu$ M
Primer A (50 $\mu$ M)	0.5-1 $\mu$ M

Primer B (50 $\mu$ M)	0.5-1 $\mu$ M
HotStarTaq Polymerase (5 u/ $\mu$ l)	2.5 u/reaction
Template DNA	Up to 1 $\mu$ g/reaction
H2O	To 50 $\mu$ l

---

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  $\lambda$ -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

<i>Pb01</i> 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  $\mu$ l 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 $\mu$ l
10X Buffer (Promega)	1 $\mu$ l
dATP (10 mM)	0.2 $\mu$ l
Taq DNA Polymerase in Buffer B (Promega)	1 $\mu$ l
H2O	To 10 $\mu$ l

---

The mixture was incubated at 70°C for 15-30 minutes. After cooling on ice, 2  $\mu$ l 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 electrophoresis. 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 Promeiga'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 DH5 $\alpha$  (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  $\mu$ l of competent cells mixed with 2  $\mu$ l 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<sup>+</sup> 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 RNeasy lysis buffer (Ambion) and stored at -80°C.

2. On the day of RNA extraction, RNeasy lysis buffer 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  $\mu$ l 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  $\mu$ l of the total RNA on a 1% agarose gel followed by ethidium bromide staining.

#### mRNA Extraction

1. For each 250  $\mu$ g of *Pb01* total RNA, the volume of the sample was adjusted to 500  $\mu$ l with RNase-free water, then 500  $\mu$ l Buffer OBB and 20  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ g of mRNA on a 1% agarose gel and ethidium bromide staining.

### **2.10 Construction of $\lambda$ -ZAP-cDNA Library of *Pb01***

The  $\lambda$ -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 *Eco*RI adapters ligated to the blunt ends. *Xho*I digestion released *Xho*I and *Eco*RI cohesive ends to allow the cDNA to be inserted into the Uni-ZAP XR vector in a sense orientation (*Eco*RI-*Xho*I) with respect to the *lacZ* promoter. 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<sup>-</sup>McrB<sup>-</sup> *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  $\lambda$ -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  $\mu$ l of 10 $\times$ first-strand buffer

3.0  $\mu$ l of first-strand methyl nucleotide mixture

2.0  $\mu$ l of linker-primer (1.4  $\mu$ g/ $\mu$ l)

31  $\mu$ l of mRNA (7  $\mu$ g)

1.0  $\mu$ l of RNase Blocking Ribonuclease Inhibitor (40U/ $\mu$ l)

3.5  $\mu$ l of MMLV-RT (20 U/  $\mu$ l)

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

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

20.0  $\mu$ l of 10 $\times$ second-strand buffer

6.0  $\mu$ l of second-strand nucleotide mixture

116  $\mu$ l of sterile distilled water

2  $\mu$ l of RNase H (1.5 U/ $\mu$ l)

11  $\mu$ l of DNA polymerase I (9 U/ $\mu$ l)

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

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

23  $\mu$ l of blunting dNTP mix

2.0  $\mu$ l 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  $\mu$ l of *EcoRI* adapters. The following components were added to the tube containing the blunt-ended cDNA and the *EcoRI* adapters:

1.0  $\mu$ l of 10 $\times$ ligase buffer

1.0  $\mu$ l of 10 mM rATP

1.0  $\mu$ l of T4 DNA ligase (4 U/ $\mu$ l)

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

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

1.0  $\mu$ l of 10 $\times$ ligation buffer

2.0  $\mu$ l of 10 mM rATP

6.0  $\mu\text{l}$  of sterile water

1  $\mu\text{l}$  of T4 polynucleotide kinase (10 U/ $\mu\text{l}$ )

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  $\mu\text{l}$  of *Xho*I buffer supplement

3.0  $\mu\text{l}$  of *Xho*I (40U/ $\mu\text{l}$ )

5. Size fractionation. Fractionation of large size cDNA increases the quality of the cDNA library. 50- $\mu\text{l}$  of a *Xho*I digest was mixed with 50  $\mu\text{l}$  H<sub>2</sub>O and 2  $\mu\text{l}$  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  $\mu\text{l}$ , 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  $\mu\text{l}$  of sterile water. The concentration of the cDNA was determined to be 40-50 ng/ $\mu\text{l}$  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  $\mu\text{l}$  of resuspended cDNA (about 100 ng)

0.5  $\mu\text{l}$  of 10 $\times$ ligase buffer

0.5  $\mu\text{l}$  of 10 mM rATP

1.0  $\mu\text{l}$  of the Uni-ZAP XR vector (1  $\mu\text{g}/\mu\text{l}$ )

0.5  $\mu\text{l}$  of T4 DNA ligase (4 U/ $\mu\text{l}$ )

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

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

1  $\mu$ l of a dilution

200  $\mu$ l of XL1-Blue MRF' cells at OD<sub>600</sub> 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  $\mu$ l of 0.5 M IPTG

50  $\mu$ l 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  $\lambda$ -Uni-ZAP-cDNA library. Aliquots containing 10,000 plaque forming units (pfu) of the library were mixed with 600  $\mu$ l of XL1-Blue MRF' cells, which were grown in LB supplemented with 10 mM MgSO<sub>4</sub> and 0.2% maltose to an OD<sub>600</sub> 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  $3 \times 10^8$  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  $\mu$ l of mRNA sample (1  $\mu$ g mRNA)

1  $\mu$ l of SMART IV oligonucleotide

1  $\mu$ l of CDSIII/3' PCR Primer

Deionized H<sub>2</sub>O was added to make a total volume of 5  $\mu$ l. The tube was incubated at 72°C for 2 minutes then cooled on ice for 2 minutes. The following were added:

2  $\mu$ l of 5X First-Strand Buffer

1  $\mu$ l of DTT (20mM)

1  $\mu$ l of dNTP Mix (10 mM)

1  $\mu$ l 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  $\mu$ l of Sodium Hydroxide and incubating at 68°C for 30 minutes.

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

11  $\mu$ l of First-strand cDNA

71  $\mu$ l of Deionized H<sub>2</sub>O

10  $\mu$ l of 10X Advantage 2 PCR Buffer

2  $\mu$ l of 50X dNTP Mix

2  $\mu$ l of 5' PCR Primer

2  $\mu$ l of CDSIII/3' PCR Primer

2  $\mu$ l 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  $\mu$ l of proteinase K was added to 50  $\mu$ l 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  $\mu$ l deionized H<sub>2</sub>O.

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

79  $\mu$ l of cDNA

10  $\mu$ l of 10X *Sfi* Buffer

10  $\mu$ l of *Sfi* Enzyme

1  $\mu$ l of 100X BSA

The digestion was incubated at 50°C for 2 hours. Then 2  $\mu$ l 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  $\mu$ l of deionized H<sub>2</sub>O.

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

1  $\mu$ l of cDNA

1  $\mu$ l of pDNR-LIB

0.5  $\mu$ l of 10X Ligation Buffer

0.5  $\mu$ l of 10 mM ATP

0.5  $\mu$ l of T4 DNA Ligase

1.5  $\mu$ l of deionized H<sub>2</sub>O

The ligation was incubated at 16°C overnight.

7. Transformation. The ligation mixture was transformed into XL10-Gold Kan<sup>r</sup> Ultracompetent Cell (Stratagene) and plated out on LB-Chloramphenicol (30  $\mu$ g/ml) plates. About  $1 \times 10^6$  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  $\lambda$ ZAPII-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  $\lambda$ ZAPII-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<sub>2</sub>O

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<sub>600</sub>=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<sub>600</sub> of 1.0

250 µl of purified plaques (>1×10<sup>5</sup> phage particles)

1 µl of the ExAssist helper phage (>1×10<sup>6</sup> 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<sub>600</sub> 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 (*DraI*, *EcoRV*, *PvuI* and *StuI*). 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  $\mu$ l of genomic DNA (100 ng/ $\mu$ l)
- 10  $\mu$ l of 10X Restriction enzyme buffer
- 10  $\mu$ l of Restriction enzyme (10 U/ $\mu$ l)
- 57  $\mu$ l of Deionized H<sub>2</sub>O

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  $\mu$ l of each reaction on a 0.5% agarose gel. The remaining 95  $\mu$ l of digested DNA was purified by phenol extraction and concentrated by precipitation with sodium acetate and ethanol. The pellet was dissolved in 20  $\mu$ l of TE buffer (pH7.5).

#### Ligation of GenomeWalker Adaptors

For each library construction, the following components were added:

- 4  $\mu$ l of digested DNA
- 1.9  $\mu$ l of GenomeWalker Adaptor
- 1.6  $\mu$ l of 10X Ligation buffer
- 0.5  $\mu$ l of T4 DNA ligase (6 units/ $\mu$ l)

The reaction was incubated at 16°C overnight followed by termination at 70°C for 5 minutes. For each construction, 72  $\mu$ l 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  $\mu$ l of GemoneWalker library DNA

5  $\mu$ l of 10X Extensor Buffer

1  $\mu$ l of dNTP (10 mM each)

1  $\mu$ l of gene specific primer (50  $\mu$ M)

1  $\mu$ l of AP1 or AP11 or AP3 (10  $\mu$ M)

0.5  $\mu$ l of Extensor Hi-Fidelity PCR Enzyme Mix (5U/  $\mu$ l)

To 50  $\mu$ l of Dionized H<sub>2</sub>O

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  $\mu$ l of the product was analyzed on an agarose gel.

### Secondary PCR

The primary PCR product was 1:50 diluted in H<sub>2</sub>O 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  $\mu$ M. 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  $\mu$ l of 10X Mutazyme II reaction buffer

1  $\mu$ l of 40 mM dNTP

X  $\mu$ l of template (100 ng target gene, not the whole plasmid)

0.5  $\mu$ l of forward primer (50  $\mu$ M)

0.5  $\mu$ l of reverse primer (50  $\mu$ M)

1  $\mu$ l of Mutazyme II DNA polymerase (2.5 U/ $\mu$ l)

To 50  $\mu$ l of H<sub>2</sub>O

#### 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  $2 \times 10^4$  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. cerevisiae* 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<sub>600</sub>=0.2. Cells were incubated at 30°C for several hours with shaking at 250 rpm until the OD<sub>600</sub> reached 0.5. Cells were centrifuged at 1,000xg for 5 minutes at room temperature, and then washed with sterilized H<sub>2</sub>O. 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 <u>PEG/LiAc</u>	6 ml of <u>PEG/LiAc</u>

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  $\mu$ l of the plasmids isolated were used to transform NovaBlue *E. coli* competent cells, which were then subjected to either Amp<sup>r</sup> selection for pGADT7 constructs or Kan<sup>r</sup> 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

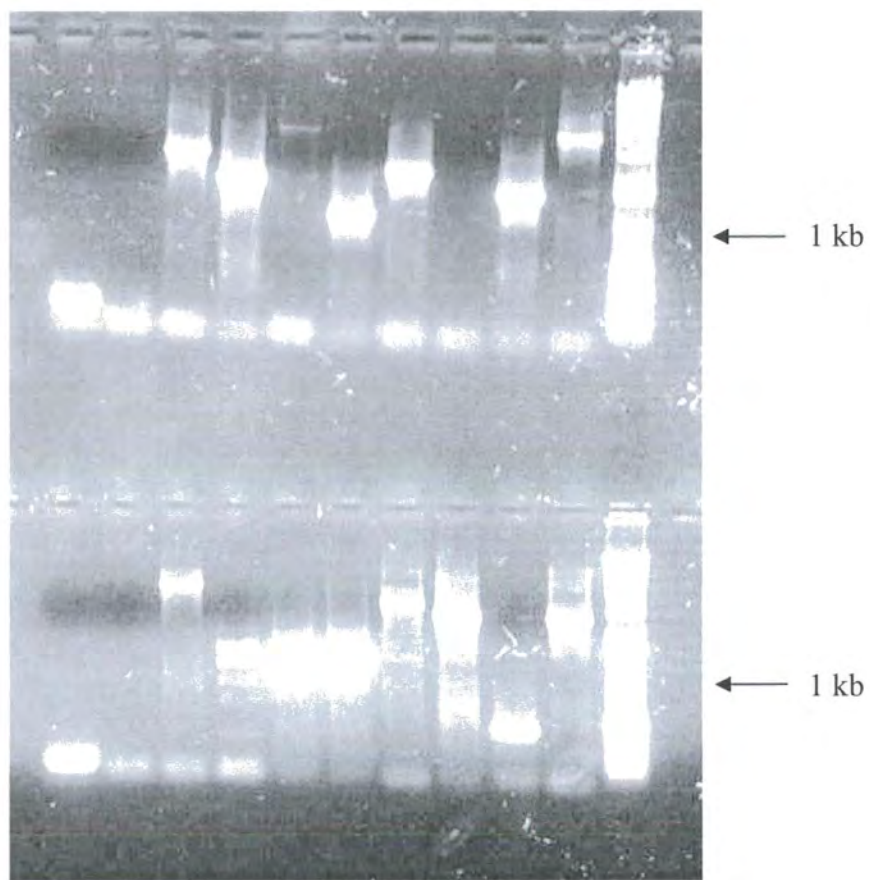
#### 3.1 Construction of Two cDNA Libraries of *Pb01*

*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 electrophoresis 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  $\lambda$ -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  $\lambda$ -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  $\lambda$ -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  $\lambda$ -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*  $\lambda$ -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-Pb01cDNA 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.

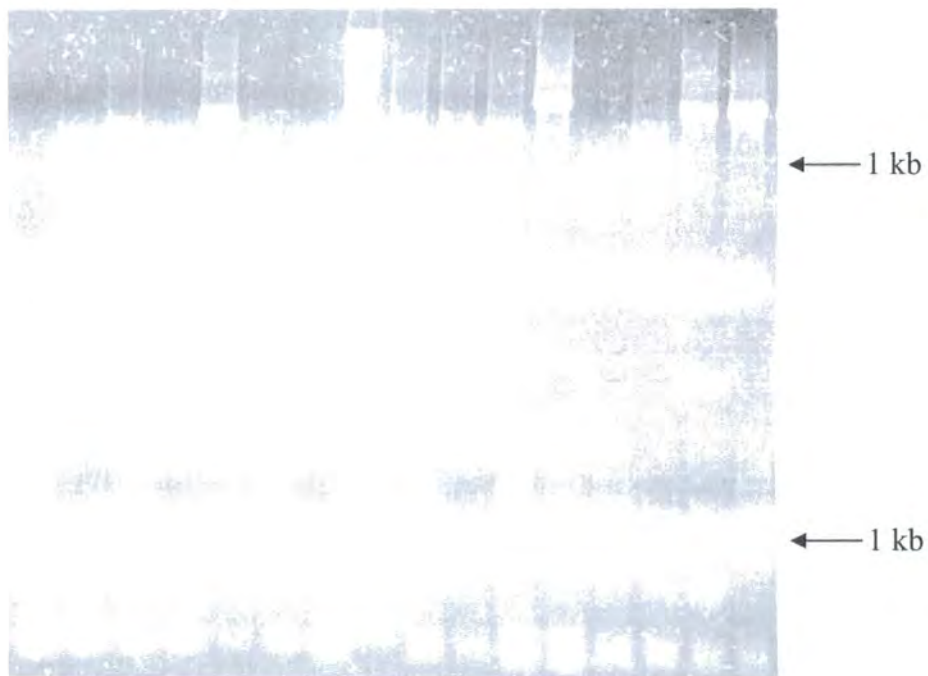


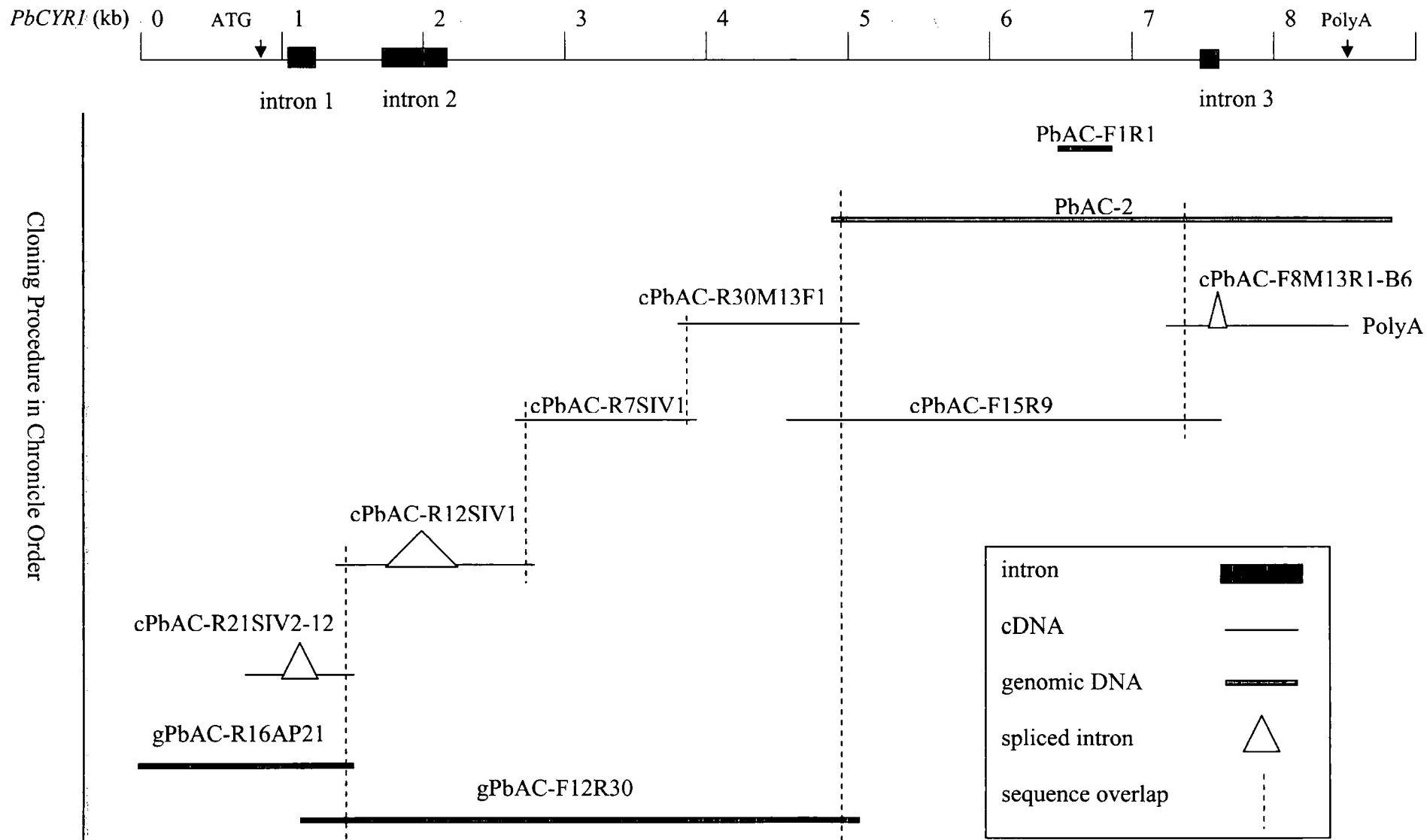
Figure 3.1-1, the average size of the cDNA insertions was more than 1 kb. The packaged  $\lambda$ -ZAP-cDNA library was amplified. The amplified  $\lambda$ -ZAP-cDNA library contained  $3 \times 10^8$  plaque forming units/ml. It was stored at  $-80^\circ\text{C}$ .

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  $\mu\text{g}/\mu\text{l}$ . The library plasmids were stored at  $-20^\circ\text{C}$ .

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

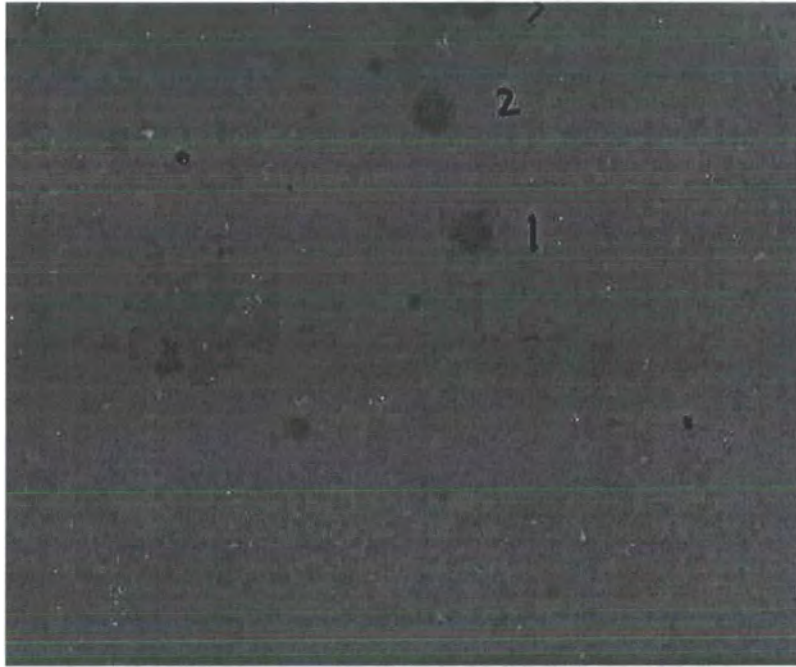
The strategy for cloning the adenylate cyclase gene *PbCYR1* included library screening and PCR amplifications walking on both genomic DNA and cDNA. Primers were designed on the basis of immediate sequencing results. The cloning procedure is described as following and illustrated in Figure 3.2-1. Initially, 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  $\lambda$ -ZAP-cDNA library. Approximately 6,000 plaques from the  $\lambda$ -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  $\lambda$ -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.

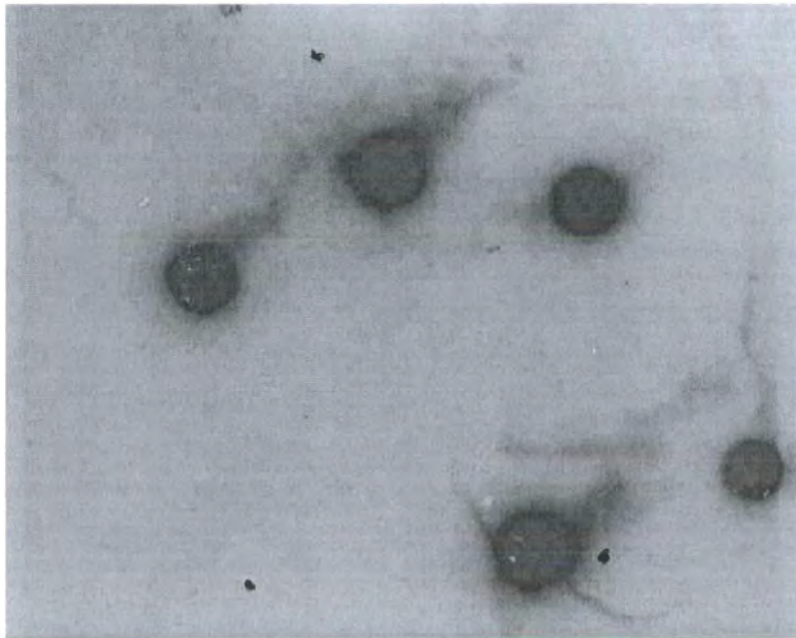


**Figure 3.2-1 Cloning of *PbCYR1***

**Figure 3.2-2. The first (upper panel) and the third screening (lower panel) of  $\lambda$ -ZAP-cDNA library with a fluorescein 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 2<sup>nd</sup> 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  TATGTGTGTATGATTCCTTCGAACCCCCCTTCTTGGTGATGAGCATGGC
-1350  AGTGCTACAGCTAGAGGGGGACCTGTAGCATTCGATCCCTACCCCCGATA
-1300  TACGGCCAAGTACACCTAGGCTTCGGCAGGAACATTGTCACCGCGAGTTA
-1250  AAGTCCGGAGTTGACGGTCGGCATCTCCTCTCTCGGGTTTGACATTAATA
primer PbAC-F23
-1200  AGTGGGGAATTGAGGTTGCGGTCACGCTACTGTTAGTTAGTCGAACTGGC
-1150  AGGGGAACTCTAGCTCATGGTCCCAGCGTTAACATCCCAGACAACCTCTG
-1100  GCGCGAATGCAACGGTTGGAGTCGGAGAGGTGTCTGTGGCTCTGTCTGTG
-1050  GCTCTGTCTGAGATTGTATACTTGATTGCTGGGTTGCGATTTCAGGCTGAA
-1000  CATCTACTTGGCCCCCTCCTGTGATTCATATAGTGATCATATACTCCAGA
-950   ATTATACTCCAGAATTAGGGAGTTAAATTATGTTGACGTGAGAGAGAGTC
-900   CCGTGCCTGACACAGCCTTGAGTAAGCGGACACGTGGGTCTAGCGCTTG
primer PbAC-F22
-850   CTTTCGGGACAGCCGAACCGGCCCCATCGTTAGTCAGCAGCTGCCGGAA
-800   CGGCGTTGTTTCGCTGTTTGGCGGGCTGGACGTTGCTCTTTCGGGCTTGG
-750   ATGGGTCATTTCACTCTATCCCGATGTTATCATAAGTCAAACCATCATCA
-700   GTCTTCGAACATTATGCTTTAAACACCACCTCCCCATTCCCCAACATAAC
-650   TGCCTGCTTCATGCTGCTTGGGGGTTTCTTCCGTCGTATGAACGGATGAA
-600   GCATAGCTCCTTATTTTAGCCTCTTTGTCCGCCTACCCCCGTCCCCTCG
-550   CCTAGCTCTTACCCCGTTGTCCTTCCTTTGTCCCCGTATGTCCGGATATA
-500   TATGCACACACTGCAGGTTCTGAAATGGTTTAGCTTTGGGATACCGTCTG
-450   CTCTTCTCTCGCCATCTGTCTCGTCGCTGTGTATCCCGTGGCCAACG
primer PbAC-F21
-400   GATTACGTTTGGAGCCAATGAGCCAGTTGGTTTACCTTCGTAGCCTTTC
-350   CTAGATTTTCGTATCCACGTATCCCCAACCCGGGATTTGAGCTACTCACA
-300   GAGTTCATATTTCGACCCCGAGCTTCGTGGCACTTCAAATCATATCTCCCC
-250   TACATCCCCCATCCAAGTCCCTTCCACGTCTCAATTCCACCCGCCACCCG
-200   CCTTGTCCAACCCAACATCTATCTACGCTGAATATCGGCCGGATGAATCA
-150   CATCCACTCGCGTCGTCTGAGAGTGTGAGAGGCCGAAAGCTTCAACCECCC
-100   GTCCAGCTCTCCATTCCCCCCCCATCCCTTTCCCCCCCCCCCCCTTCCAA

```

-50 CGGGGCTTTTGTATCTCCTTGGAGCGGCCAGAAACTTAGATTTGCGGCAGC  
**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 AGGGTCTTGGCTTGAAAGGGAACCCCGTCGCCCCAGTAGAGCTAGGGAG  
34 Q G L G L E R E P R R P S R A R E  
151 ATTTCCGATCAGGATGACAGgtatgtttgatttcgttttgaagaagcag  
51 I S D Q D D  
201 ttttgcttcgtagatgaattgtaggtcctgtttgggcacatcggaggcct  
251 tcattctgttcaaaatgtgggaaacgctgttacgcaaacgttttataact  
301 taatTTTTTTTTctttccagAAGGCTGAACTCGACATCCAGACACTCGTC  
57 R R L N S T S R H S  
351 GTCCTCAAAGAGTAATCGTAACGCCCCCGTAGGCCACATTCCCCCGATG  
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 ACAATACTCAAGCACAAATGCTCGGAAGCGGTGCGCCATCATGGACACCAC  
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 TCTGATGCGGAGAAACCTCCCCTCAATTTTCAGCTGGAGGCTGCCCTAAC  
151 S D A E E T S P Q F Q L E A A L  
651 CGATCCCAGTAATTTCCCAGAACCCGTCCATGACGAACATCGCAGACCCT  
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 GGTCTGTTTCCACAAAACGCTGCGCGGTCTCTTTGGGGATGAGCATGGCAG  
201 G R F H K R L R G L F G D E H G  
801 CGCGTCAGATTGCGCTCTCCCGGAGCGGAGTCTGTCAAACCGAATTCCTA  
217 S A S D S A L P E R S L S N R I P  
851 TGTCATCTGGCAGTCAATCAAAGgtccggtgaagaaataattcgacccca  
234 M S S G S Q S K  
901 tctttgcacccccagagcgttcttcacccttggtcgtggcttccgacc  
951 atgcactcctctacettccagtgatggttacccttgaatgtatcaaaagc  
1001 tcaatctaagatggcacgaaaagttccttcgttaatcgggaatttacttca  
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1101 cggaaagtcttctgaaggtatctggataaaaactgcacgacttggtgaaac  
1151 tgcataattatcatcactagctctataaacctttctcccttcattattt  
1201 ggtttgcccatccctttctctaccattcctggctctctattttctaattag  
1251 caccctcagggacattttcattatattctcgccatttcattctgaatta  
1301 tactcgaactttaagctaattgtgggattgtagGATATATCAAACCTATGGA  
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

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 GGGCTAACTTGCAACACATTTTCAGATCTTCAGACCATGACAACCACAA  
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1551 ACAAGTGTATAGTAAAGTAAAAGAGGGCACCCCTCTCTCCAACATATCCCCA  
314 K Q V Y S K V K E G T L S P T I P  
1601 CCGCTCTAGGAGCCCCGCTCGATCAGTCCTTCGCCTAGTTTGCAGGGCGGC  
331 T A L G A R S I S P S P S L Q G G  
1651 AGTGGCAAGACTCTCAAGGGACAAGGTCGCCTGGAGGTTATCCAACAA  
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1701 GCGATCATTTTTTACGAAGATTCGACGCCATCCAAAACCTCCAATCGGGA  
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1801 AAGGGATCTAGTTTTTTGAAACGAGATATGTCTCCTGCTAGATATGGTTCG  
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1851 GAATTCGAGCTTTTGATAGTGTACCAATACAAAGCCGTGGGAAAGTGTTCG  
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431 E S D R K K E S A K G V S K F R H  
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448 P R F P F G H D T G N K D D K T  
2001 CCAAGATAATTTTGAACAAGATCGGATCTGGAAGTTAGATACCGATCTGA  
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2101 TGTGGTGGTATATATACTGGAACCTCCACTTCCTGAGCATAAGGAAAGAGA  
498 C G G I Y T G T P L P E H K E R  
2151 ATTCCGCAATCCCTTTGAACCCTCCAGCACTGGTACTGGCATGCTCCAG  
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 CTGTGTCCGCATCTTTCGCATCGACTCTACATTTACAACCTTTTCCTCTA  
564 Y C V R I F R I D S T F T T L S S  
2351 ATCTGAACACTACCGTGACCGAAATACTGGAATGCTTGGCCGCAAGTCG  
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  
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614 D L S R T L D Y G E R P I L M Q K  
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631 K L L E Q A G Y Q P S D R I A D I  
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681 Q K F S H V D L H G R S L I T L P  
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698 I T L Y K R A S E I I S L N L S  
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2901 TGAACAGCTGGATAATGCAGAATTACACAATTTGCATGGACTTGTTCAGCT  
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798 F P A L R S L N I S S N S Y R T  
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3101 TCAACAAAATCTCCGAGTTGTCGGATATTGGACGCCTTACGACACTGGAA  
831 F N K I S E L S D I G R L T T L E  
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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 TCTTGCCCTGGACCATTGCCAGTCACCGAGTTTGATCTCACTAGCCCCC  
914 T L A L D H C P V T E F D L T S P  
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931 L P T L T S L N I A S A K L V Q F  
3451 AAGGATACCCTTTTTACCAATATCCCACACCTGACCAAACCTTATTCTTAA  
948 K D T L F T N I P H L T K L I L  
3501 TAAGAATCATTTTCGTTACTCTCTCCTCCTATATCGGAACCCTCCGAAAAC  
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3551 TTGAGCACTCAGCATTTTCGAAAAATCCCCTGTCAACTCTTCCACCGACG  
981 L E H F S I S K N P L S T L P P T  
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998 I G C L T E L K C L N L R E C N  
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1031 N V S S N V L D A F P K I A G S P  
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1064 P G L A S P S H E E L G K L E D F  
3851 AAGCCAGACGACCAAGTCATTCCTCCAGTGGTCTTCTGAGCGTGGCAAGT  
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4251 CTCGGACGACCTTGAAGAATCAAGTAGTCTAAAGGTATTGCACATAAACG  
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4301 GCAATAAATTCAGTACTTCTGCGGAGCTCTGTAAGGTGAATCGCCTT  
1231 G N K F Q V L P A E L C K V N R L  
4351 GCGATTTTATAGATGTGGGGAGTAATTCTCTAAAATACAATGTTTCCAACCTG  
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  
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1281 F S G N K R F E I K P N T S Y T S  
4501 GGGATTCCCTGCCGTTAATAGCACAGATCTGACCAACTTCAACTCTTTGAA  
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  
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1331 I P D Q T E D R R V R T S S S L S  
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1348 G S L A Y G M A D S L G R N E H  
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1364 L S I V D M L V T R F R G N Q T E  
4751 CGGTTCTCGGTTTATTTGACGGCCAGTCCCTCTTCTAGCAGCGGATCAAGG  
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1398 I A K Y L Q E H F T T S F T E E  
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1448 M D D R E L H H F E R G T T T S  
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1581 E L W D Y V T P D V V V D V A R S  
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1598 E H G D L M I A S Q K L R D L A  
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1641 I A Y G A T N K I M V M I A G V S  
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1648 M G L S P L T D E H L F T T P S  
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1664 K I K K R G R P G D S R L A R L D  
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1698 K S T S L W E T Y P V A M R S A  
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1714 I Q I H N D L F R R Q L A Q I G G  
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1731 F E V K T E G D A F M V S F S T A  
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1748 T A A L L W C F T C Q S Q L L E

5901 GGAATGGCCGACGGAGATCATGGAATCTCCTGCTTGCAGGCCACAGTATG  
1764 A E W P T E I M E S P A C R P Q Y  
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1781 D S D D N I I Y R G L S V R M G V  
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1798 H W G T P V C E Q D P V T G R M  
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1814 D Y F G P M V N R A S R I S A V A  
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1831 D G G Q I F V S A D F V A E I Q R  
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1848 N L E T F A D Y D R I G S M D S  
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1914 H S L A G R L N A P R A E I E E A  
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1948 D L V W R L W T V A L R L E A F  
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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  
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1998 V L S L I E H Q V T R V E  
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6701 aaaccogttatagACATGTGCAAATACCCTCACAATCCGGCACATGATGA  
2011 T C A N T L T I R H M M  
6751 GGCCTTTCAAACGCGGAAACTCTCTCAAAGATATCGCCGTTCCCATGTG  
2023 R P F K R G N S L K D I A V P M S  
6801 GATATTCTCAATCAGTTGAAGGTGCAGTTGAGCGAATTTAGAGCTCAAA  
2040 D I L N Q L K V Q L S E F R A L  
6851 GGAGTCAATAAAGTCCCATCGCCGACTCTCAGGATCCCCAACAAAGCCTTG  
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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 TTCTTTTATGCTCTTACTCAACTAGCGCCGATCGGATTGCAAGTCGTCGG  
  
7101 GAGTTATTCTCTGTTCCACCCTTTTTTTTTTCGCTTCCCCTTTTTCTCTT  
  
7151 TCGTCCGCTTTTGTCTCTTTATCCCGGCCGTTGCCTATGACTTGATGTC  
  
7201 CGTTTGATTTAATTCGTGGGAAAAGCAGCGTATTTGGAATTTAATTACC  
  
7251 ACCTTGCCGTATTACTTTCTGCCATATTTTGGTTTATTTTTTTCATTAC  
  
7301 TTTATTTACTTAATTTTGGGTGTGGGGGGTTTTCGCTCTGTTTGGTGGTA  
primer PbAC-R32  
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7401 CGTTCATTTCTGGGATTACACCATTTTATTTTTGCTAAGATCGGCGTATG

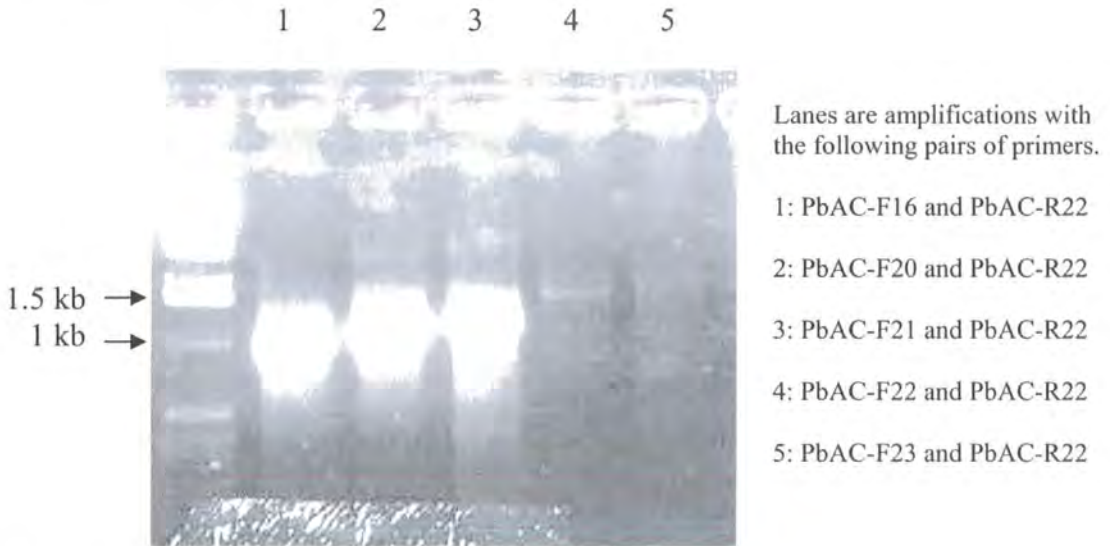


**Figure 3.3-2. Determination of transcription initiation site of *PbCYR1*.**

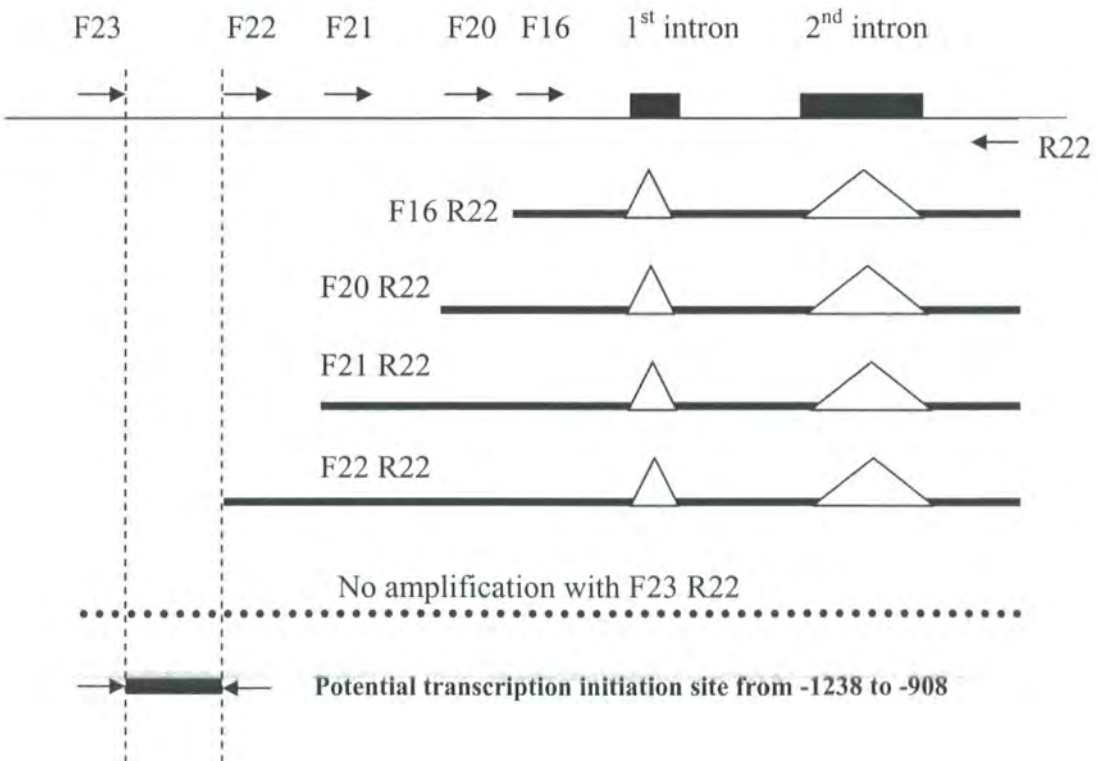
**A:** RT-PCR products after amplification with different pairs of primers. Bands can be observed when using primer pairs “PbAC-F16 and PbAC-R22”, “PbAC-F20 and PbAC-R22”, “PbAC-F21 and PbAC-R22”, and “PbAC-F22 and PbAC-R22”. No band was amplified when using “PbAC-F23 and PbAC-R22”.

**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**



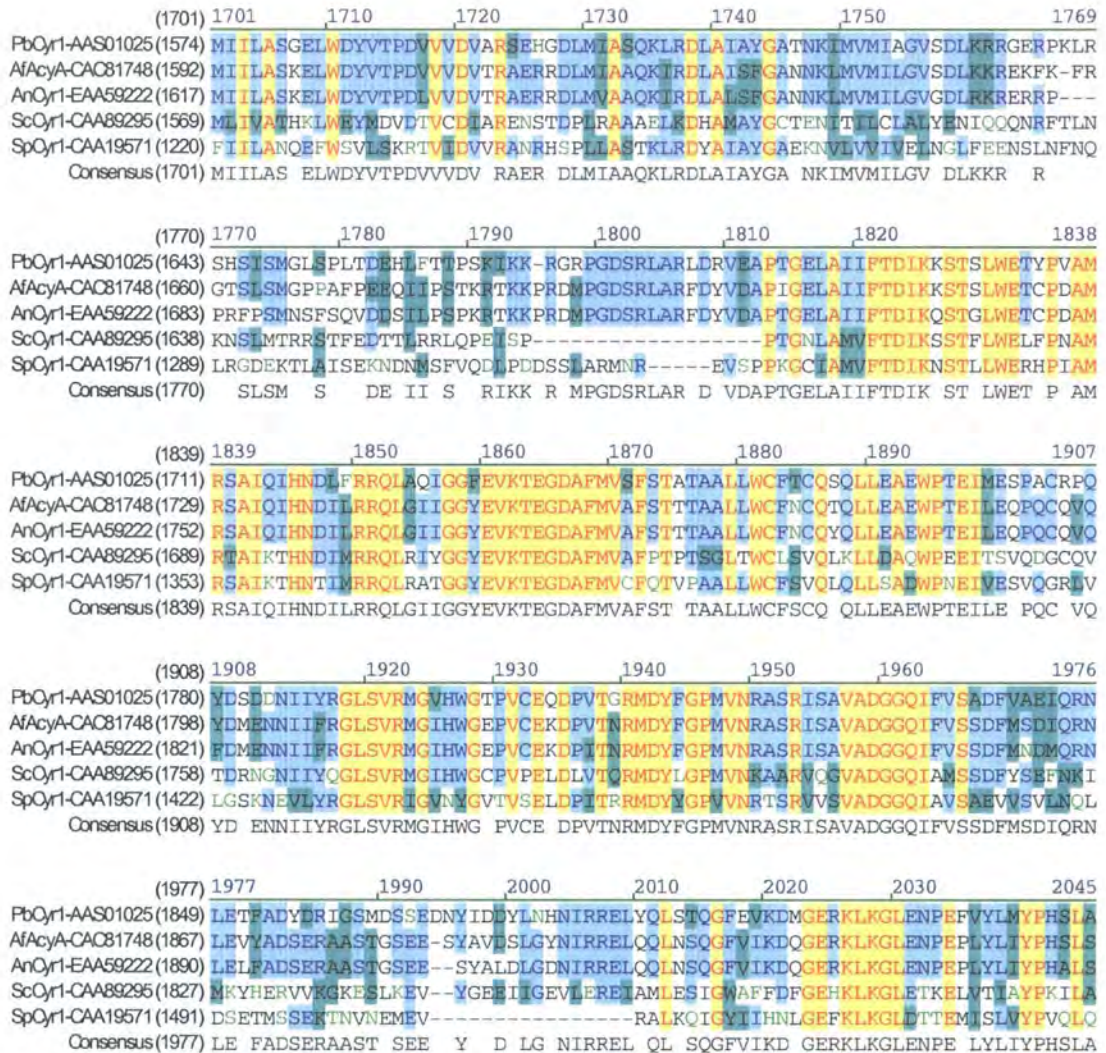
**B: Schematic illustration of A**



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

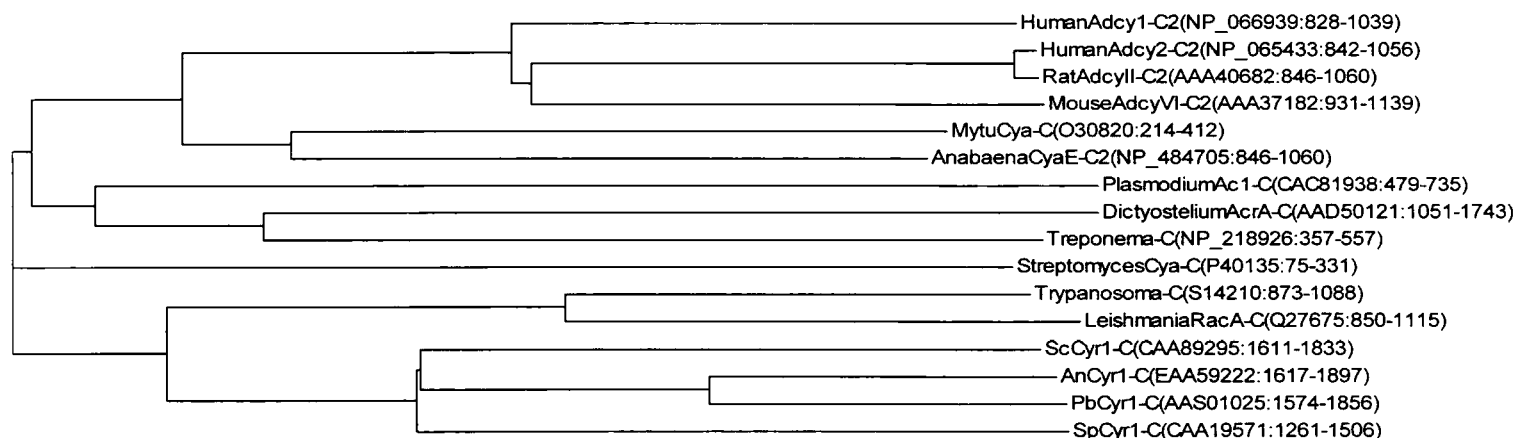
<b>GenBank Accession Number</b>	<b>Protein: Organism</b>	<b>% identity to PbCyr1</b>
AAS01025	PbCyr1: <i>P. brasiliensis</i>	
CAC81748	AcyA: <i>A. fumigatus</i>	53%
EAA59222	AnCyr1: <i>A. nidulans</i>	50%
AAC34139	Mac1: <i>M. grisea</i>	39%
XP_329423	NcCyr1: <i>N. crassa</i>	34%
BAA93553	CaCyr1: <i>C. albicans</i>	27%
P49606	Uac1: <i>U. maydis</i>	24%
CAA89295	Cyr1: <i>S. cerevisiae</i>	24%
EAL19815	CnCyr1: <i>C. neoformans</i>	24%
CAA19571	SpCyr1: <i>S. pombe</i>	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. brasiliensis*; Af, *A. fumigatus*; An, *A. nidulans*; Sc, *S. cerevisiae*; Sp, *S. pombe*.



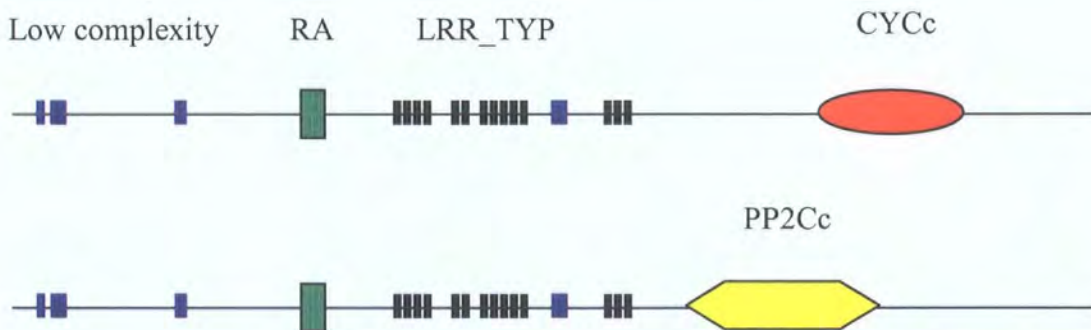
**Figure 3.3-5. Phylogenetic relationship of Class III adenylate cyclases.** The tree was constructed by using Vector NTI 6.0 (Informax). Only catalytic domains were used in the phylogenetic analysis (for mammalian adenylate cyclases, it is the 2<sup>nd</sup> catalytic domain that was used). The protein 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 tuberculosis*; Anabaena, *Anabaena* sp. PCC7120; Plasmodium, *Plasmodium falciparum*; Dictyostelium, *Dictyostelium discoideum*; Treponema, *Treponema pallidum*; Streptomyces, *Streptomyces coelicolor*; Trypanosoma, *Trypanosoma 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-Fidelity 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 1<sup>st</sup> and the 2<sup>nd</sup> intron, suggesting differential splicing in those regions. To delineate the 1<sup>st</sup> and the 2<sup>nd</sup> 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 1<sup>st</sup> 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<sup>Δ58</sup> and PbCyr1<sup>Δ58-64</sup>.

**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).

	(160)	160	170	180	190	200	210	221
PbCYR1-genomic DNA	(160)	CAGGATGACAG	GGTATGTTT	TGATTTTCGTT	TTTGAAGAAGCAG	TTTTCGTT	CGTGATGAAT	TGT
PbCYR1-cDNA-R21SIVI-12	(160)	CAGGATGACAG	-----	-----	-----	-----	-----	-----
PbCYR1-cDNA-R20SIVI-8	(160)	CAGGATGACAG	-----	-----	-----	-----	-----	-----
PbCYR1-cDNA-R21SIVI-2	(160)	CAGGATGACAG	-----	-----	-----	-----	-----	-----
Consensus	(160)	CAGGATGACAG	-----	-----	-----	-----	-----	-----

	(222)	222	230	240	250	260	270	283
PbCYR1-genomic DNA	(222)	TAGGTCCTGTT	TGGGCACAT	CGGAGGCC	TTCATTCG	TTCAAAA	TGTGGGGAAAC	GCTGTTA
PbCYR1-cDNA-R21SIVI-12	(171)	-----	-----	-----	-----	-----	-----	-----
PbCYR1-cDNA-R20SIVI-8	(171)	-----	-----	-----	-----	-----	-----	-----
PbCYR1-cDNA-R21SIVI-2	(171)	-----	-----	-----	-----	-----	-----	-----
Consensus	(222)	-----	-----	-----	-----	-----	-----	-----

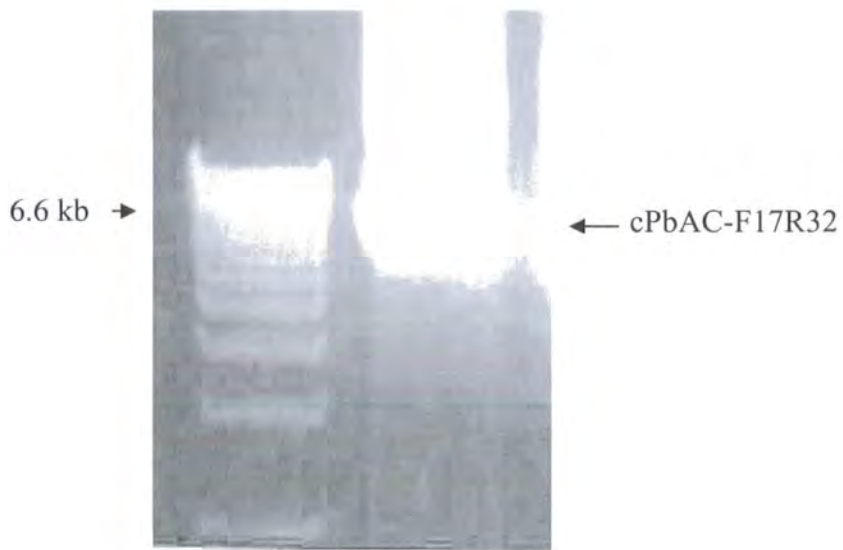
  

	(284)	284	290	300	310	320	330	345
PbCYR1-genomic DNA	(284)	CGCAAACGTTT	TATACTTAAT	TTTTTTTTT	TCTTCCAG	AAGGCTGAACT	CGACATCCAG	ACAC
PbCYR1-cDNA-R21SIVI-12	(171)	-----	-----	-----	-----	AAGGCTGAACT	CGACATCCAG	ACAC
PbCYR1-cDNA-R20SIVI-8	(171)	-----	-----	-----	-----	GCTGAACT	CGACATCCAG	ACAC
PbCYR1-cDNA-R21SIVI-2	(171)	-----	-----	-----	-----	-----	-----	ACAC
Consensus	(284)	-----	-----	-----	-----	AAGGCTGAACT	CGACATCCAG	ACAC

**B:** Differential splicing leads to the 2 types of truncations for PbCyr1 protein, i.e. PbCyr1<sup>Δ58</sup> and PbCyr1<sup>Δ58-64</sup>.

	(30)	30	40	50	60	70	84
PbCyr1 (R21SIVI-12p)	(30)	LLISQGLGLERE	PRRPSRAREI	SDQDDR	LNSTSR	HSSSSKSNR	NAPRRPHSPDV
PbCyr1 (R20SIVI-8p)	(30)	LLISQGLGLERE	PRRPSRAREI	SDQDDR	LNSTSR	HSSSSKSNR	NAPRRPHSPDV
PbCyr1 (R21SIVI-2p)	(30)	LLISQGLGLERE	PRRPSRAREI	SDQDDR	-----	HSSSSKSNR	NAPRRPHSPDV
Consensus	(30)	LLISQGLGLERE	PRRPSRAREI	SDQDDR	LNSTSR	HSSSSKSNR	NAPRRPHSPDV

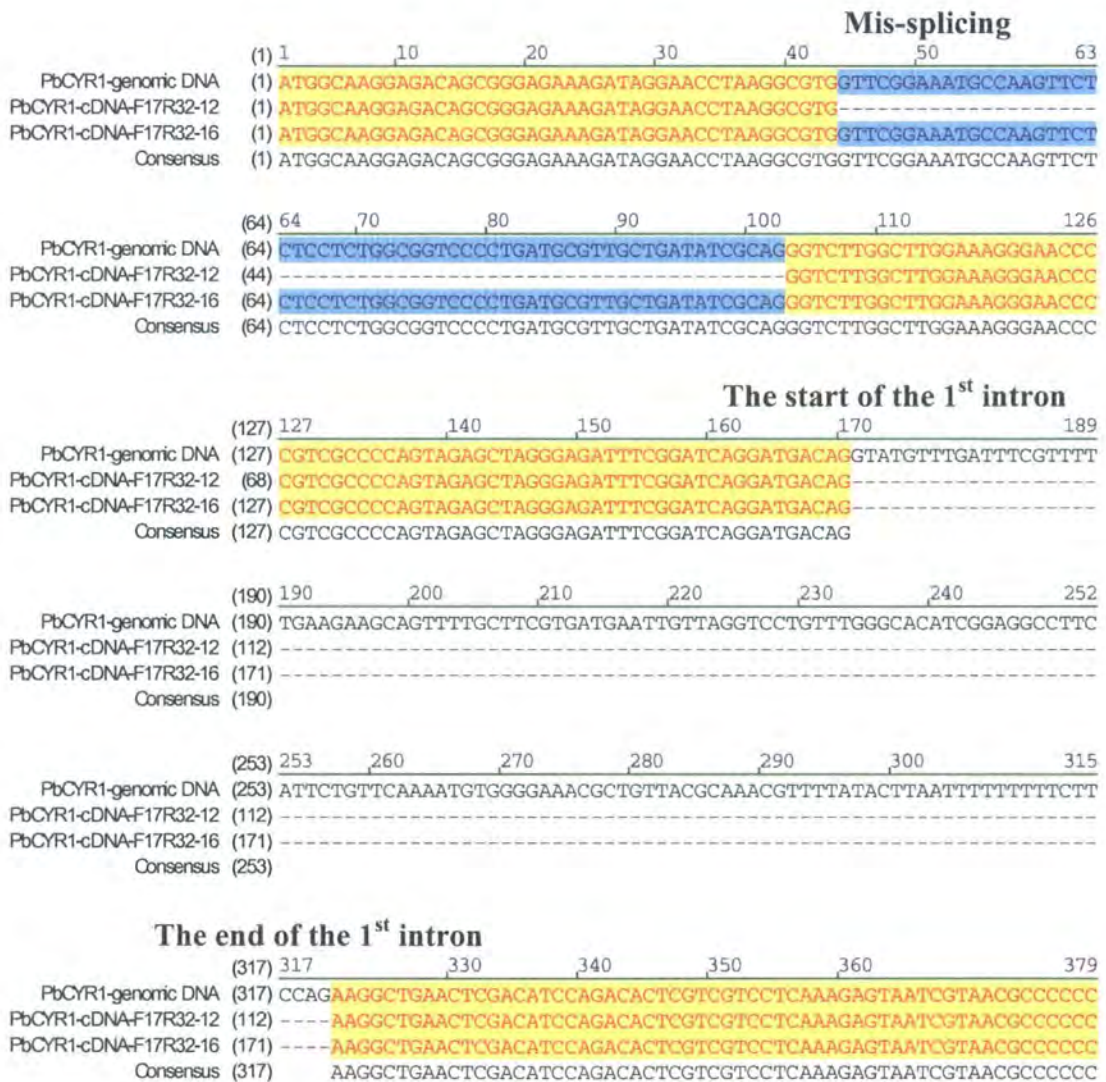
**Figure 3.4-2 Full-length *PbCYR1* cDNA amplification by RT-PCR.** RT-PCR was done by using primer PbAC-F17 (positions -29-1) and PbAC-R32 (positions 7332-7364). A single band of 6.6 kb cDNA was obtained. Further analysis of the band indicated differential splicings at the 1<sup>st</sup> and 2<sup>nd</sup> 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 (positions 171-320).

For clone PbCYR1-cDNA-F17R32-16, the 2<sup>nd</sup> 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 2<sup>nd</sup> intron

(830) 830 840 850 860 870 880 892  
 PbCYR1-genomic DNA (830) **GTCGTCAAACCGAATTCC**TATGTCATCTGGCAGTCAATCAAAGGTCCGTGAAAGAAATAATT  
 PbCYR1-cDNA-F17R32-12 (621) **GTCGTCAAACCGAATTCC**TATGTCATCTGGCAGTCAATCAAAG-----  
 PbCYR1-cDNA-F17R32-16 (680) **GTCGTCAAACCGAATTCC**TATGTCATCTGGCAGTCAATCAAAG-----  
 Consensus (830) GTCGTCAAACCGAATTCC**TATGTCATCTGGCAGTCAATCAAAG**

(893) 893 900 910 920 930 940 955  
 PbCYR1-genomic DNA (893) CGACCCCATCTTTGCACCCCCAGAGCGTTC**TACCCCTTGGTCGTGGCTTCCGACCATGCA**  
 PbCYR1-cDNA-F17R32-12 (665) -----  
 PbCYR1-cDNA-F17R32-16 (724) -----  
 Consensus (893) -----

(956) 956 970 980 990 1000 1018  
 PbCYR1-genomic DNA (956) CTCTCTACCTTCCAGTGATGTTACCCCTTGAATGTATCAAAGCTCAATCTAAGATGGCAGC  
 PbCYR1-cDNA-F17R32-12 (665) -----  
 PbCYR1-cDNA-F17R32-16 (724) -----  
 Consensus (956) -----

### New exon caused by mis-splicing

(1019) 1019 1030 1040 1050 1060 1070 1081  
 PbCYR1-genomic DNA (1019) AAAAG**TTCC**TT**CGTTAATCGGAATTTACTTCAAGGGAAGCCTATATGGTTTCC**TGTCGTTACA  
 PbCYR1-cDNA-F17R32-12 (665) -----**TTCC**TT**CGTTAATCGGAATTTACTTCAAGGGAAGCCTATATGGTTTCC**TGTCGTTACA  
 PbCYR1-cDNA-F17R32-16 (724) -----**TTCC**TT**CGTTAATCGGAATTTACTTCAAGGGAAGCCTATATGGTTTCC**TGTCGTTACA  
 Consensus (1019) AAAAG**TTCC**TT**CGTTAATCGGAATTTACTTCAAGGGAAGCCTATATGGTTTCC**TGTCGTTACA

(1082) 1082 1090 1100 1110 1120 1130 1144  
 PbCYR1-genomic DNA (1082) **TGTTC**CCAACTGCT**TCCTTCGGAAAGTCTTCTGAAGG**TATCTGGATAAAACTGCACGACTTGT  
 PbCYR1-cDNA-F17R32-12 (723) **TGTTC**CCAACTGCT**TCCTTCGGAAAGTCTTCTGAAGG**-----  
 PbCYR1-cDNA-F17R32-16 (782) **TGTTC**CCAACTGCT**TCCTTCGGAAAGTCTTCTGAAGG**-----  
 Consensus (1082) **TGTTC**CCAACTGCT**TCCTTCGGAAAGTCTTCTGAAGG**

(1145) 1145 1150 1160 1170 1180 1190 1207  
 PbCYR1-genomic DNA (1145) TGAAACTGCATATTATTCATCACTAGCTCTATAAACCC**TTTCTCCCTTCATTATTTGGTTTGC**  
 PbCYR1-cDNA-F17R32-12 (760) -----  
 PbCYR1-cDNA-F17R32-16 (819) -----  
 Consensus (1145) -----

(1208) 1208 1220 1230 1240 1250 1260 1270  
 PbCYR1-genomic DNA (1208) CCATCCCTTCTCTACCATTCTGGCTCTCTATTTCTAATTAGCACCCCTCAGGGACATTTTC  
 PbCYR1-cDNA-F17R32-12 (760) -----  
 PbCYR1-cDNA-F17R32-16 (819) -----  
 Consensus (1208) -----

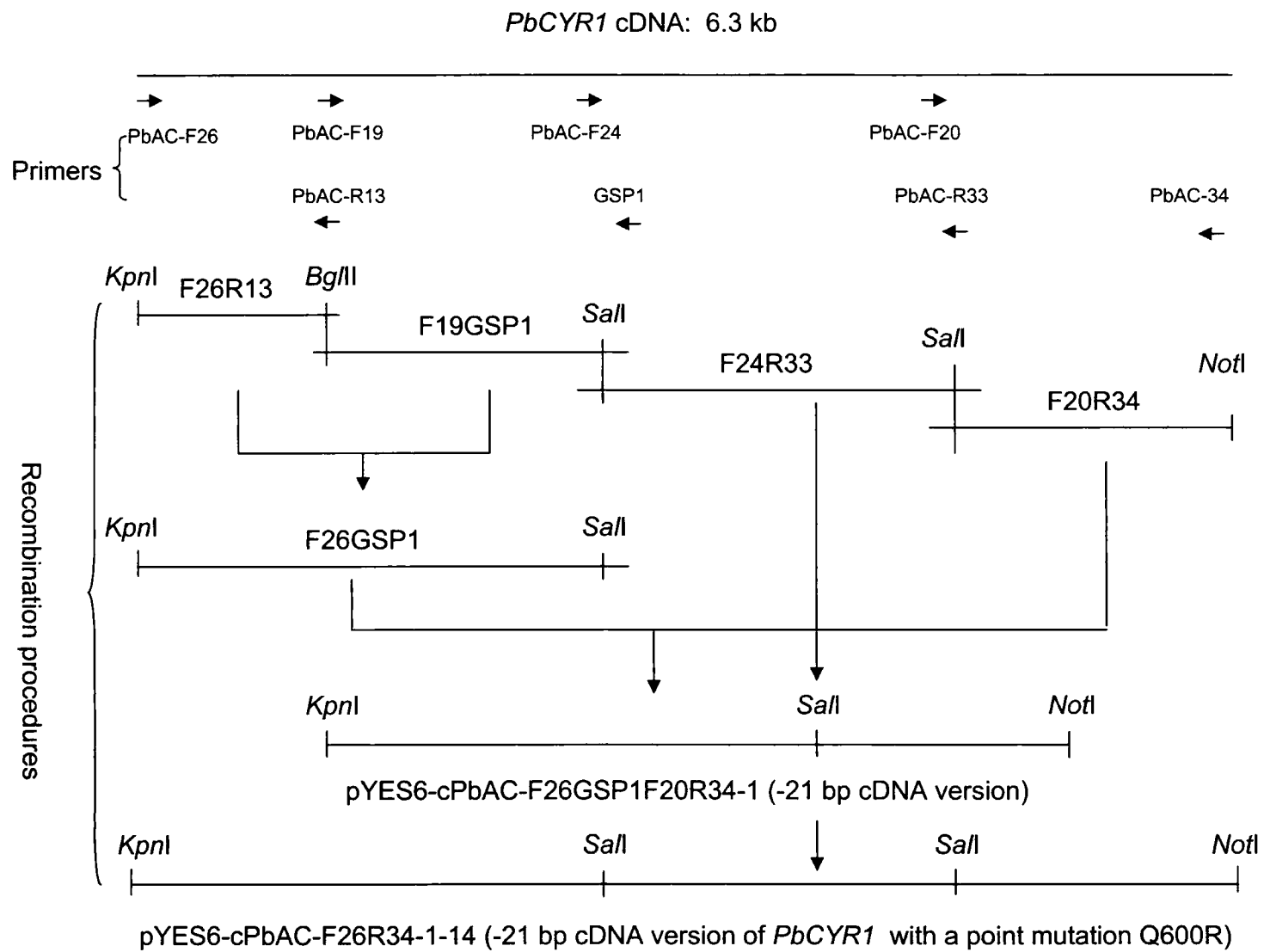
### The end of the 2<sup>nd</sup> intron

(1271) 1271 1280 1290 1300 1310 1320 1333  
 PbCYR1-genomic DNA (1271) ATTATATTTCTCGCCATTTCACTTCTGAATTATACTCGAACTTTAAGCTAATGTGGGATTGTAGG  
 PbCYR1-cDNA-F17R32-12 (760) -----  
 PbCYR1-cDNA-F17R32-16 (819) -----  
 Consensus (1271) -----

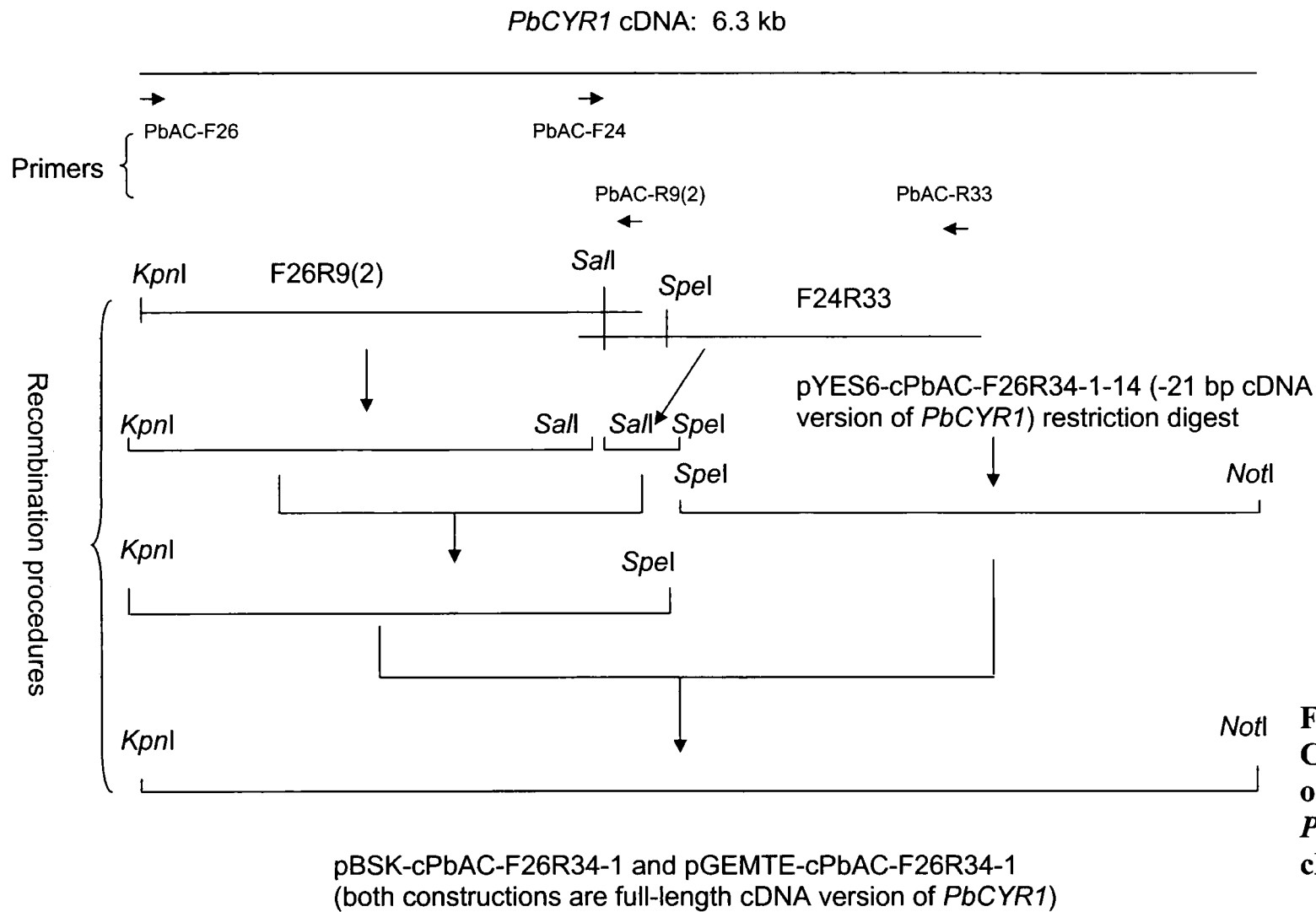
(1334) 1334 1340 1350 1360 1370 1380 1396  
 PbCYR1-genomic DNA (1334) **ATATATCAAAC**TATGGAGATGCACCTGTGTATCCAGT**TCCAGGAGACCCGGATGTTGAACGCT**  
 PbCYR1-cDNA-F17R32-12 (760) **ATATATCAAAC**TATGGAGATGCACCTGTGTATCCAGT**TCCAGGAGACCCGGATGTTGAACGCT**  
 PbCYR1-cDNA-F17R32-16 (819) **ATATATCAAAC**TATGGAGATGCACCTGTGTATCCAGT**TCCAGGAGACCCGGATGTTGAACGCT**  
 Consensus (1334) **ATATATCAAAC**TATGGAGATGCACCTGTGTATCCAGT**TCCAGGAGACCCGGATGTTGAACGCT**

### 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 *KpnI* 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**

### 3.6 Cloning of 3 Iso-forms of G Protein $\alpha$ 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.

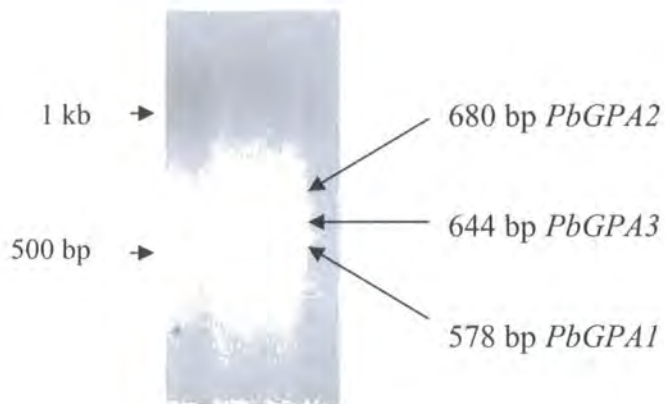
On the basis of the gPbGPA-DGF5R2 (578 bp, *PbGPA1*) sequence, two specific primers, PbGPA1-F1 and PbGPA1-R2, were designed for upstream and downstream SSP-PCR. By using these 2 specific primers and 2 anchor primers (M13R1 and M13F1), 2 cDNA fragments, namely cPbGPA1-F1M13R1 and cPbGPA1-R2M13F1, were 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

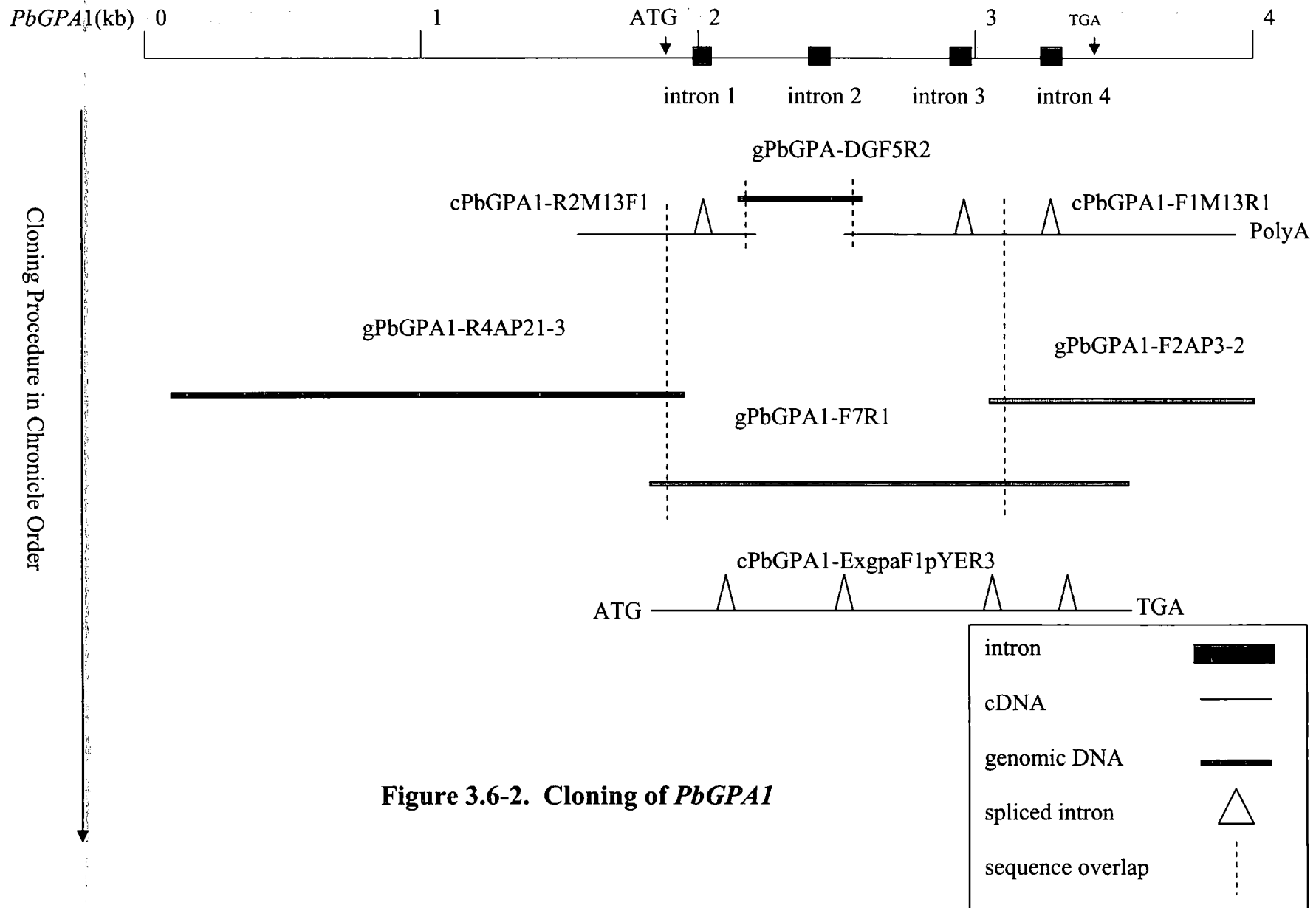
codon and TGA stop codon respectively. The cloning procedure for *PbGPA1* is illustrated in Figure 3.6-2.

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 GenomeWalker 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

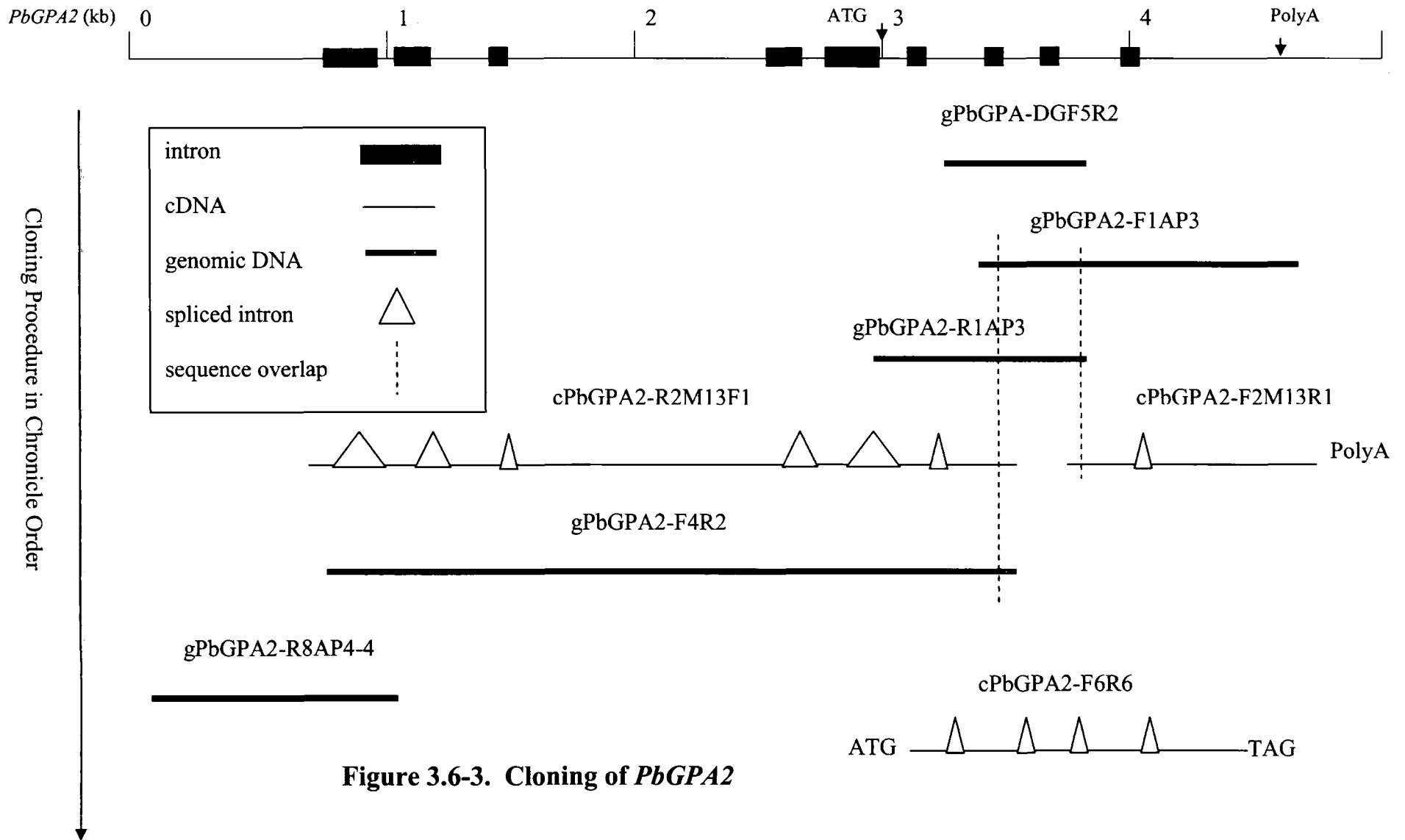
**Figure 3.6-1. Three iso-forms of G protein alpha subunit amplified by using degenerate PCR.** With a pair of degenerate primer PbGPA-DGF5 and PbGPA-DGR2, 3 iso-forms of *PbGPA* were amplified from *Pb01* genomic DNA.



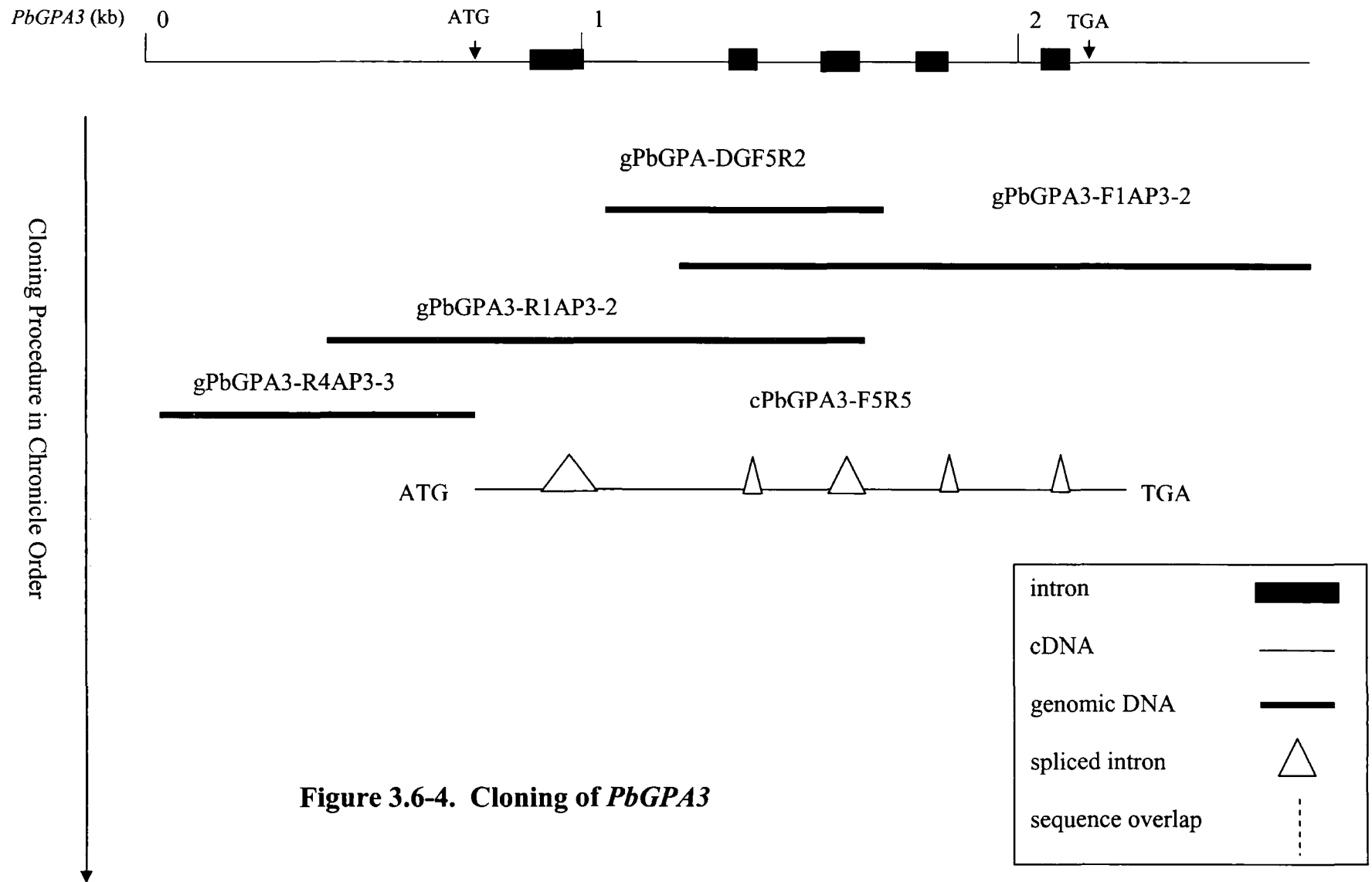


Cloning Procedure in Chronicle Order

**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 $\alpha$ 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  $\alpha$  subunits can be grouped into 3 distinct subfamilies (Figure 3.7-5). The close relatives of PbGpa1 are as the follows:

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

The close relatives of PbGpa2 are as below:

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

The close relatives of PbGpa3 are as below:

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

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

```

-1950  TTTAAGCTATACAGGGTCAAGTTTCCTTCCAAGCCTTCTAGAAAGCATCA
-1900  CGATTGCACTGGGCTCCACAAGTGAAGAGGGGGTGGAAACGAAAGGCTGG
-1850  TAAATTTGGGATTTTTGTAGGGAATGGCTTGGAGGAGTTTAACCCTAGGC
-1800  GAACGGAACAATTATTTTTGGAAGGGAATGGCTTGTCTATGACTCGTGGG
-1750  TTTTCGCCTCTTGTCTGGGCTGTTGAACTGGGATGATGAGGATGACATG
-1700  CAAGGTAGACTTGTCTCTAATATCTCCTCCTGTGGAGATATAAATTTCT
-1650  GATTAAATTTCTCCCAATTTCTCCCAATTTCTTCCGTTTTTCGTTTTCCCC
-1600  TCTGGCGGGATTGGGTTCCACTCTGCAGTCAAGCTGCCGATTCGTCAGCG
-1550  GGATAAACATGGGTGCAAGAACCATTTCAACCTTTTTTCCGTTTATCTCT
-1500  AAAGCCCAGCTTTGGGAAGTTCTTCTATTTTCGGGACACTGCGTCCTCT
-1450  ATTTATCGATGTTGATACGCTGGGAACAATGGATGCCACGTTTGCCTGAG
-1400  CTTACCTAGGCAGAAAGGTATCCAACGGGCAAATTTCTTATCTTTCTCG
-1350  AGAAATTTCCGGACGTATTTCTAAAGGGGCACACAAACGCAGGCAGACTG
-1300  CAGTGCAGTATTATCTCCAAGGCTGGGAGTGGATTAATAATTTGATACCC
-1250  AAGAAACTAATAATGTCATCCTAATAAGCCATGGGTAATTGCGCCCGTTA
-1150  TAGTTACCTCTCCTCCCCCTTTATTTTTATTTCTTACACGAGATGGGGGAC
-1100  TTGTAAGGAAGAGACCAATTTCTTGGGCGGTGCGTGGGTGGGTATTCTGGG
-1050  GTGTTCTGTTGTACGAAGCAGGTCACCAGCGGACTCATTTCCAACCAACT
-1000  CGGCCATGGATGGAGGAGAAAACCTGCCCTTGTACTGCACATACTAGTGT
  -950  ATTTAGCACTTGACACCTCGTTTGGCGCTGGACCTTGGCGTGTGTTGCAA
  -900  GGCGGTTGAATATTGTATGTATCATAACTTATTGAACTGTAACATAACAG
  -850  CCGTAAAGGCCCGTGACCATACCGTACAACGCTTCTCTTCCCTCGTCCCA
  -800  CCTTCCCCCTTCTCAGCCGGCATAACCTTGTGTGTCTTTTCTGCTTCTT
  -750  CCTCTCCATTGTTCTTTGCCAGGCCAACCTCCAAACCTCCAAACCTCC
  -700  AACCTCCAAACCTCCTCCTTCGCTATCTTCATCTCCATCTTCACCTTCAAT
  -650  CCTCTAATCTCCCCGCTGCGGTGTTGTTGGACTGTTGGCCTGAACCTGT
  -600  AGGATTCCATTCCAGTCCTTTCCCGCAAACCCAGCCAGTCTTCAGCAAA
  -550  TTGAATCGCTCTTTTCCAGCGTGCGATTTGGTCCGCTCTCCCTAAGTT
  -500  CTCTTTGACTCCCTGCAGACCACCACTACCTGACCTCTTCTGCTCTGCCC
  -450  ACCAACCCCTCTCTCTTTGGAATACGGTATTATCGGTATCTCCAAAGCAAT
  -400  ACTGAATTGAGTTGATTGGTCTTGGTTAATTGTTTCTTTCCCCCATTGG
  -350  AGTATATTGTGAGTGTAGAATGTCTTGACTTTATGGCTTGCCTCTTGGGC
  -300  TCTTGTGCCGATTGTGCGGACGACGGCATTGGGGGAATGTGACCCGGCT
  -250  GCAACCCCTTTGTCTGCCAGCAGCAACCATCTTCTACCAATCGATTTC
  -200  ACTCTCTCTTTACCGCCCTACCTGCTTTTTTTTTACCTTGTGAGCTCTGG
  -150  GATGTGTAGTTGATCCATTTCCCACTGACATGAAGCAGAACCTGTACATT
  -100  CCGATATCGTCCAGAGCATTTTGTGTCATTCATCCATCTTGCTTTACGCC
  -50  CTGAAAAATAAACTCACCCAGTCTTTACACATTCTGTAACCTGACAACA

      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  TCAAAATGCTCTTGCTTGgtaagatgattcctttttccttttgtttaatcc
     34   I K M L L L
    151  ggctgtatgacttacttggctctgaaccaccagGTGCTGGAGAGTCTGGC
     40                                     G A G E S G
    201  AAATCCACAATTTTGAAACAGATGAAACTCATTACGAGGGTTCATATTC
     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  GATGACCAGCGTGCCGAATACCATGTTTCAGACTATCTTCATGCAACCAGC
     96   D D Q R A E Y H V Q T I F M Q P
    401  ACAGATTGAAGGCGATTGCTTACCCCCGATGTTGGCAGTGCAATCGACG
    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

```

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 TCTCACGTACCGTATGTTTCGACGTAGGTGGTCAGCGTTCTGAGCGCAAGA  
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 GCAGGAAGCTCTCACACTTTTCGATTCCATTGCAATTCGAGATGGTTTA  
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 AAACTGCCTGTTAGCCCAATGAAAACTACTTCCCTGATTACGAAGgttt  
277 K L P V S P M K N Y F P D Y E  
1001 gttttcgcccaactcctttatttttctcttattcatgataaacgttgctg  
  
1051 atattggtgaccagGGGGTGCCGAATACACTGCTGCTTGCGACTATATCC  
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 TGTCAACGgtatgtggcttgcttcattttttatgtacaaattaactaga  
337 A V N  
1251 atcatacatgtcatgctaactctagagtcagATATAATCATCCAGGACA  
340 D I I I Q D  
1301 ACCTTCGCCTCTGTGGACTGATATGACGAAGTCTTTTGAATACGTTTGAT  
346 N L R L C G L I \*  
1351 TCTTTTTAGTTACTTTTTGATTTTTTTTCGCTAATTAGATAATTTTCCTTTT  
  
1401 CACGCCATGTGTATTTGATATTACCTTCAGCTTTTTCTTTGTACCTTGTC  
1451 TTCCTCTTGGTTAAGGTTTATCTCCGGCCCGCTCCCTCCCTGACCTGT  
1501 CAGCATCTGTCAATTCAGAAATTCCTTTTCTATTAAGACATCTATACTTTT  
1551 GCCCATATTATGGAGGATTTGACCAGTGGTTGAAAAGCCCTTTTTCTAC  
1601 ATCCCAAAAACATGGAGTAATGCAGCCTCGTCAGCACAACCGGCACCACA  
1651 TGAACGGGACATACGATTTCTTTCCCTGACCACCCTTTCTTTATGTGTCT  
1701 CTTTTTCTTCAGATCCATCTTGCCCTGATCTTGTACTTTTGTTTTTTTC  
1751 AATTACCCTGCATGGTTTCTTCTCAGTTTGATTTGAGATTATTTCTCTT  
1801 TTTTACCCTTTTTTATATCCTCTTGATGTATCTGTTGTGCGTATGAGGT  
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 TTGAGAAATATAAATTGCTAAGTATAAGNTTTTATTAAAAGATATATACAA  
 -2950 CTTTGATAAAAATTGAACNTGCTATGAAAGTTTATATCATTTGCTAAAAATA  
 -2900 TTACTAGTAGTGAGTGCTACNGTAGAGCAAAAGCTTTGATCAAATTATTA  
 -2850 AAATTTGTGAATATAAAAATGATCAGTATTATTATTTATGAAAAAGTAAT  
 -2800 ATCNGAATATATTCNATATAAAATAAAAATAAAAAGTAAAAATGCNAGAGA  
 -2750 TCTAAATATCAAATTTAACNAAAAATAGAAGATTTAACTAGTAAAGAAGT  
 -2700 TCAAATTCCTTAGCTTTATCTGCATCATTTGCATCATTTGCAGCATTTG  
 -2650 TTGAACAGTCAATTATTCAATTTTATCAAGTTCCTTATTACTTGCATTTGC  
 -2600 TTCATGCTGATGAGCTCTACAACAATGCAGCAATTGTAATATNTTAGGC  
 -2550 ATACAAGATTAAGATGTTCTCAACTGTGTGTAATTTAATTTAATTTAATT  
 -2500 TAATTTGAGTGTGTAGCATTGAGATAAATTTGAAATAAAATCTAATAAGCT  
 -2450 GAGCTCTGGTGATTTTGGTGTGCACTCACTTGAGAGTTGTGCTCACTTG  
 -2400 GGAAATACATTAACATAACTAGTTATAGTTAGTTAACTAATAATTAACTAA  
 -2350 CTTTGGTCATTGTGTTCCGGATATTTCTTCTTGTGACCATTTAAAGAACA  
 -2300 AAGTCAATGAGTGCAAAACGGGATCTGCAAGGGAGCGTTTAGTTCAAAAGC  
 -2250 AAAGGTCCACGGGCAATAAAATATTACCAGCGCATGTCCCGTGGCATT  
 -2200 GAAAAGTGCGAAGAAAACAACGAGACCCATAACTATATTAATGCCATTA  
 -2150 TGCTTTGCAATGAATTCAGgcaagaaaattccatgcatactctatagacaa  
 -2100 ttctttcatcgggacaacacactgaacagaggggaagGACTCGAGCTACGTT  
 -2050 GCAACGGAACAATTCCCATGTCCCTTCTCTTCTCCTGCAGGCCACACACA  
 -2000 GAATCTCCCGCTATCGATATCTCATCAACATGCCCTGGCAGTGTGTGAAAC  
 -1950 GCATAATATCGGGGCTGGATCTTAAAGATAACCACATGGCAACCAAAAGC  
 -1900 AAGCCGgtcagcattcttcttagcaacatggtcgtatcatcgggtaattca  
 -1850 agegctcatacctgtgactgggtgggtggttatttatttcttgggaagT  
 -1800 AATTTTCGTCGCCCGGCATCCCAGTCGATCACTCGCTAGTCTTGCAACAGT  
 -1750 TTTCTGGACGAATTCCAGGGCTGGTTTAGCGGGCCGTGAAGCTAATGCAG  
 -1700 CTAATCCGTTAACCAATTGTTCCCGAACTGGCGGTGTGACGCACCTGG  
 -1650 ATGCCACTGGAAGGTGGCTTGATGGCGAACTTAAACCGTCTGCCATCGAA  
 -1600 TTATTAACACACAAGgttcggtaattaccatactacataccatgggagca  
 -1550 ttagttaacactgtttcgcgatgattctgggtcagCAACCACCTCCCTCACA  
 -1500 GGGATTTGAAAGATCGAGTCCGAATGTCGGGATGTCGGCGTTCATAGAG  
 -1450 AAGATACAGCAGTCGGCGTAGAGAATCATGAGCGAGACAAGAACGTATGC  
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 -1350 AGCCGTAGGTAATAATATGCTCTGATATTGCTCTCTCCTTAGTCTCCGGT  
 -1300 AGGGGAACATGTCAATTTTCCGTCAGCGGGCCAGCTTCAAGAGTCTAGCT  
 -1250 GCAACTAAAACATAGCGGAGTCCACTACAGGATTCCTGCGCACTCCATCC  
 -1200 AACTGGCTTTGACGCTATTGAGGAAAAATAAGTAAATCACTAACAACGAC  
 -1150 CAGCTCACGCCTTTGCCGGCTGTCATGCTGTACTCATCTTTCCAACAGCA  
 -1100 GCAGCAGCAGAAGCAACAACAACAACAACACTGTTCCAATTCACCTCCTTT  
 -1050 GTATGCCTTGTTACCTGACTGGGTGCTGCTCCTCGTTCGCAGGACTCAG  
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 -950 GCCTCCTTGACCTCACGCAAACTCCCTAGCAACCGCATATCCCAATGAT  
 -900 TCAGCGGAAAAATAAACTCGTTGATACAAACCGTTAACC GGTCCTAATCG  
 -850 ACAGTAGAACTTACATACTAAATCCAAAAACTCCTCTCACTAATATTTT  
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 -750 TCCGGTGCCACTTCGCGCAGAAATACAACAGCACAGAGCAGATTCTAGC  
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 -450 CTGTTCCGGTCCGgtatgtggagccatttgtcgaettccccgcttaacta  
 -400 tgaggcaaaagttcagaatgatgggtctcatctccatgctcatgctggt  
 -350 tcatgtttaacttgcgatgctgattgagttctgatagGAATTGATTGTGCA  
 -300 TTTTAGCAGATTTCGATTAATTTGgtatgtatgctctatgeaagaetatatt  
 -250 caagttgtcaaatgtttttaacccttcccctttacgtctcatcccctata  
 -200 attttgggttctacggcaaccatggaggtctacaatctcatagcggggg

-150 **catggtacaaat**ttgatatcacttcaaaaccactttttctctcaagccatt  
 -100 **ctctgggtgatctcggattaagcctgattctttattcattttatcatctca**  
 -50 **actaatgattcattctcacgacaaaaag**GTTCCAATTCATTTCTTAATC  
  
 1 ATGGGTTGCGCAAGTTCTCAACCAGTGGATCCGACGGATAAGATTGCAGC  
   M G C A S S Q P V D P T D K I A  
 51 TAGACACACAGCTGCGATCGACAAGATGATCCGTATGGACAAGAAGGTTTC  
   A R H T A A I D K M I R M D K K V  
 101 AGGATCGAACGGTGAAGATTCTTTTGCTTG**gtacgtcactaaatctaaat**  
   Q D R T V K I L L L  
 151 **ctgcaactcgggtaatgatcatcactggaatttacttgttttaatatatta**  
  
 201 **ttcttag**GTGCTGGAGAGTCTGGAAAATCAACAATAATTAACAAATGGC  
   G A G E S G K S T I I K Q M  
 251 AATTATCCATGCGGGTGGCTTCCCCGAAGACGAACGATACCAAACCGCG  
   R I I H A G G F P E D E R Y Q N R  
 301 CGGTCACTACTCCAACATGGTTGTTGCATTCAAGATTCTCCTCGATATT  
   A V I Y S N M V V A F K I L L D I  
 351 ATGCAGACGGAAAATATTGAATTTGAGTTTGAAAAGACAAAG**gtacgaaa**  
   M Q T E N I E F E F E K T K  
 401 **tttcgaccttgattcctgctgctaaataccgacgactctggaggttttgc**  
  
 451 **ttatataaatgccgtaaag**CCACTAGCCAAGCTATTAGACCAGACAGAGC  
   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 ATGAGTGGAAATGTGGCTAGATAGGGGCGTACAGAAAGCTGTTTTACGAGG  
   M S G M W L D R G V Q K A V L R  
 601 ACACGAATTTGCACTTCATGATAATCTTCAGTA**gtacggatttctctttt**  
   G H E F A L H D N L Q  
 651 **tttttttttttgatcatggatatttcgggagatatatcaattgacata**  
  
 701 **caacacag**CTATTTCAAATCTCTCAACCGCGTCTTTACCCCTGGGTGGTT  
   Y Y F K S L N R V F T P G W  
 751 ACCCAACAATCAAGACATGCTTCATCCCCGACTTCGTACCACAGGCATCA  
   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 GGCGGGCAACGATCGGAGCGTAAGAAGTGGATTCAATTGTTTCGAGGGGGT  
   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 TTGAGGATCAAAATGCA**gtaagttcgaattcccccttttaacaaaat**  
   V E D Q N A  
 1001 **gtataaataaatggaaaaccttagcatggcttacatataattatgttgtt**  
  
 1051 **gacag**AATCAAATGCATGAAGCCATGATGCTATTTCGAATCTCTGGTAAAC  
   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  
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   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 GACCGATTTGCGGGCATCACCCGCGTCCCTGAACGGGAGATCTACATCCA  
   D R F R G I T R V P E R E I Y I  
 1301 CTACACAAATGCTACTGATACCACCCCTATTAAGGCCACGATGGATTTCAG  
   H Y T N A T D T T L L K A T M D S  
 1351 TGCAGGACATGATTATCCAGAAGAACCCTCAACAACCTGATTCTGTAGGTG  
   V Q D M I I Q K N L N N L I L \*

1401 CCAACAGTGCTAGAAATGAAACTGCAACGGAGCTCCTAGTAGGAGACAAT  
1451 TTATTTTGCCGTGATACCGAATAGAGTCTGCTATTATCACATTAAGTCTG  
1501 ACACCCCCCTCCACCGCTCCCGGATTTCTCCACGCACCCTGTCTCATCT  
1551 CCTTTTCGTTTCTTTTGATGGCTAAGATTTTTTTTCTTTTTTCTTTTTT  
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 TCTCTCTCTCTCTCTCTCTCTCTGTGGCGCTGAACGCTTCCCAATATCCT
-650 CTACCTTCTCCATCCTCGCCTTCCATTCAGCCTCTTCAACCCACCCTACC
-600 ATAACCCTGTCCAACCTCCACGAACTTCTCCCTCCTGCATTTCATCGAAGAA
-550 TTATCCCGCATCGGGCCGCTTACTTATCAAATCGACAATACCTTATTGCA
-500 ACCTACTTATTCCCAGGCCAAACTATGTGAGCCACCGTATAACTCCCCAG
-450 GCCACTCACTGCTTTCTTTGCCGATCTCGGCTTTGTGGTTCATTGTTCCA
-400 GATATCTCACGATCAACCGTCTATCCATCTGACAATCTAGCCTCCTTGGC
-350 TTCTCCATATTCCCAGAATCTATCAGAATCTACAAACTACCGTATAATAC
-300 CTTCTTCGCCTCAGTCGGTCTCCGTTCCGTGGCTTCAGGCCCAGGACGGC
-250 TGGCTGCCTCGATCTCAAATAATACACACAGTGTCCCTCCCGGTCCCATC
-200 TCGATTTGATCGCGCCTTTCCATCTATCTGCCCTTGCTACCCGAGATTTT
-150 GTTTCGAGGTTATAATCAGGCGTGTAGTGCATTCCATAATATCGTCTCCAT
-100 ACATCCATAGAGGCGCCTTACATCTCTCTCCTGTTGTGTGGTTGGTGTAT
-50 TAAGGTCATATTGTTTCGCCCTGTTAGTAGAAGCATTCGATATCAGATAGG
1 ATGGGTGGGTGTTGCAGTTCCTGCTTCTGGGGAGAATGCAGAGCAGAGGAA
M G G C C S S A S G E N A E Q R
51 GAGAAGCCAGATGATTGACAAGGGGCTCGAGGATGATTCAAACGATTAC
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 cactctggttgagtgtctgtgtgtctgttagatgtgtaaacagtggatatg
251 ttctgacgggtcattacagGTTTCGGGTGAGAGCGGAAAATCGACGATCGTA
G S G E S G K S T I V
301 AAGCAGATGAAAATTATCCATCAGAACGGATATCTGTTCGAAGAACTTAC
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 GATTACAGACCTTAgtatgccctcccccttttcactggagtcctttgcttt
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Y F F E E A K R I T

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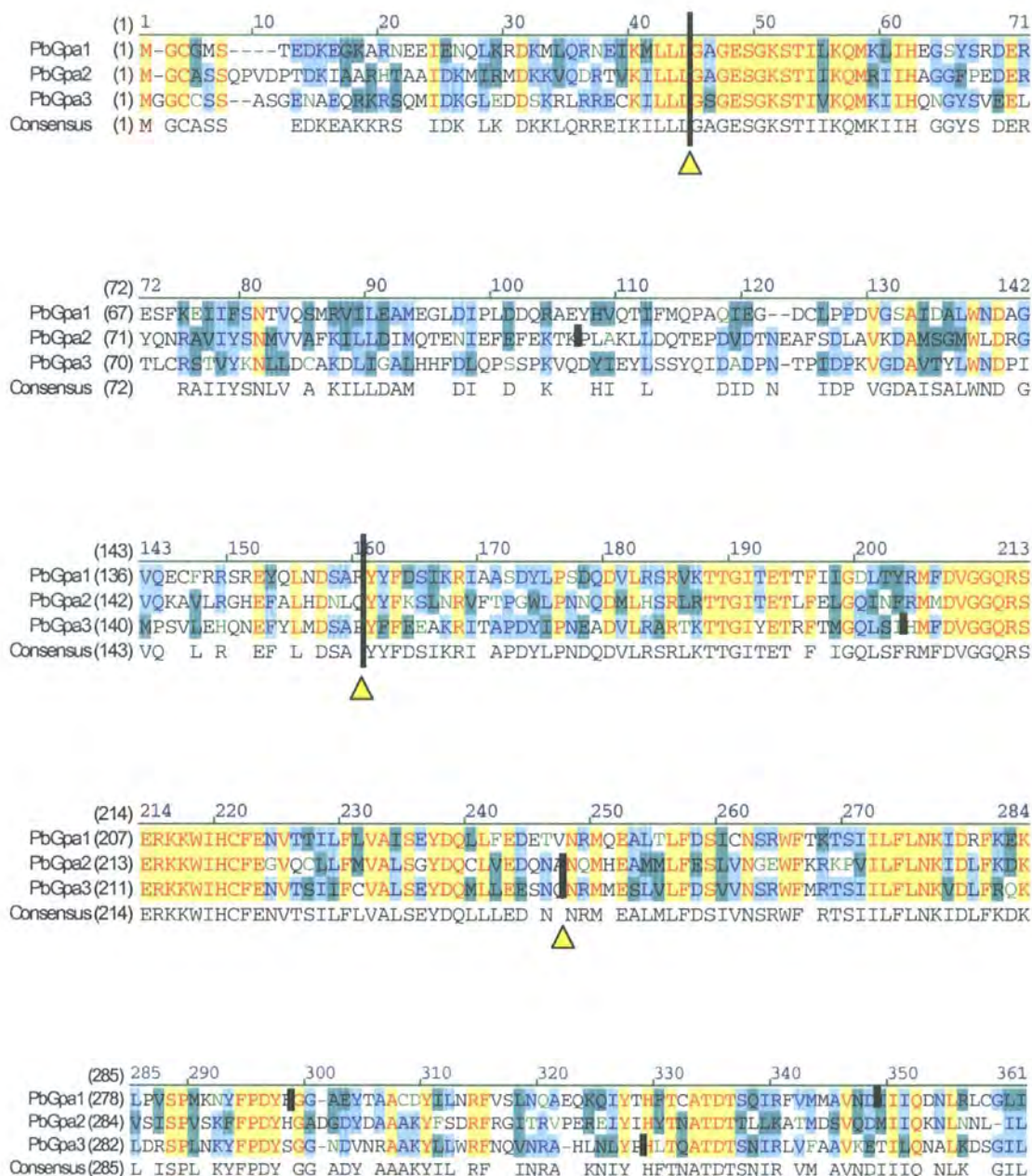
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A P D Y I P N E A D V L R A R T  
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K T T G I Y E T R F T M G Q L S I  
801 Agtgcggtttaaataatcaccctactttgcccgaacatgaccctaata  
851 gtatatcttttagCATGTTTCGATGTTGGCGGGCAGCGGAGTGAACGGAAA  
H M F D V G 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 atgaccctcatttcctcgcggttacctctattgcggttgtttactgggtcta  
1051 accttgacgaaagAACCGCATGATGGAGAGTTTAGTGCTGTTTGATTCCG  
N R M M E S L V L F D S  
1101 TTGTCAATTCCCGATGGTTTATGCGGACGAGTATAATTCCTCTTAAAT  
V V N S R W F M R T S I I L F L N  
1151 AAGGTCGACCTCTTCCGGCAAAAACCTCGACCGGTCCCCATTGAATAAATA  
K V D L F R Q K L D R S P L N K  
1201 CTTTCCCATTACTCTGGAGGAAACGACGTCAACCGTGCCGCAAGTACT  
Y F P D Y S G G N D V N R A A K Y  
1251 TGCTCTGGAGATTTAACCAAGTTAACCGGGCGCATTGGAATCTTTATCCT  
L L W R F N Q V N R A H L N L Y P  
1301 CAgtatgtatagggaaaagcacacctaattacttcttcttcttcttcttca  
1351 aaagcccttgtcctgtacctaacctccccgctgcccagCCTTACTCAAG  
H L T Q  
1401 CCACCGACACGTCGAATATTCGCCCTCGTCTTCGCAGCCGTGAAGGAAACC  
A T D T S N I R L V F A A V K E T  
1451 ATTTTACAGAACGCCCTGAAAAGATTCTGGTATTTTGTGAAAGGGAGAAAA  
I L Q N A L K D S G I L \*  
1501 CGAAGAAAACAAAGCAAAAAGGAAAACAAATACACCACGAATTGTGGAA  
1551 TTTTTTGTTCACCTTGATATTTTTTTTTTGGAAAGGCTGCGGGGGTTCGTC  
1601 TTAGCCATCGAGAGCGAATATCTATACCCCGCCGTTCCGNACCGCATTT  
1651 CCGTTTCCGCCGCAAATTATTACTTACTTGCAAATCTTCACCGGTTTGA  
1701 CTTACGATGACGACACCATTACCCACTACCCACCACCGTCTACAAAAAAA  
1751 ACGCCATAATAATGTCTTTACCCCTCTACATGCTTTTCAAACCTGTGCTT  
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Notes:

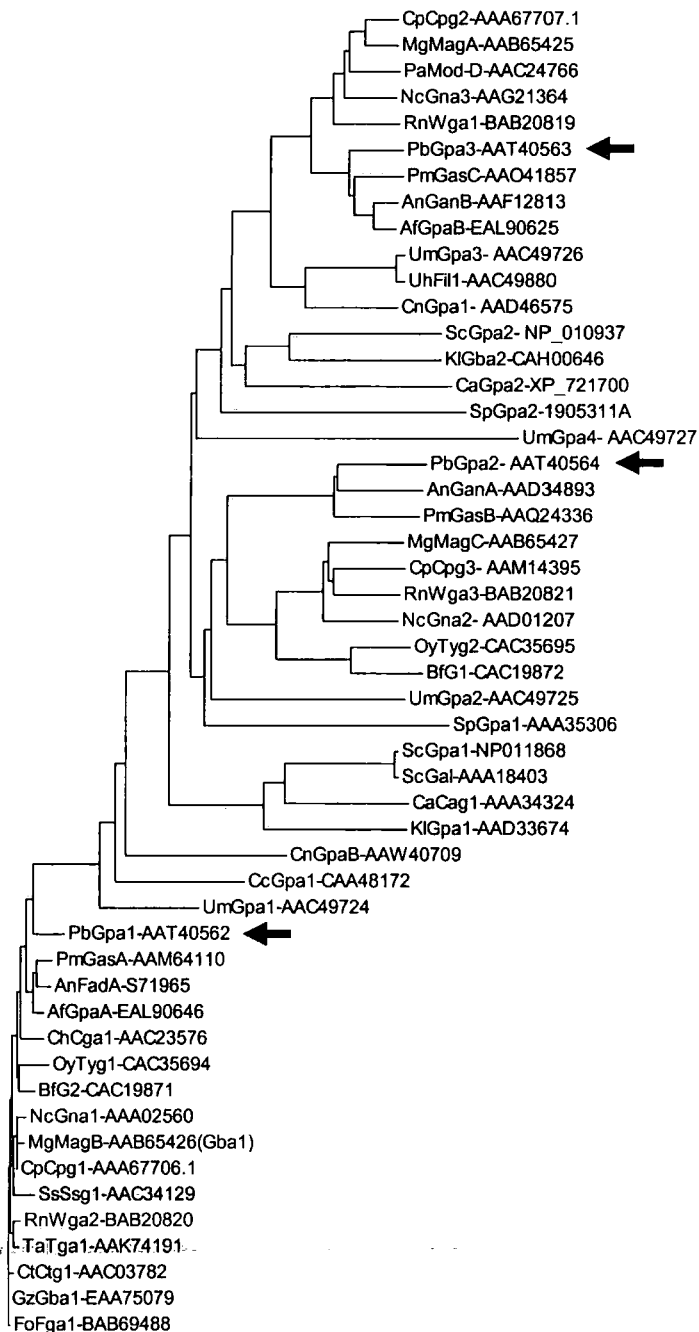
Nucleotide positions are written in red numerals, amino acids in blue.

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 Ga-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 Ga-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, *Gilberella zeae*; Kl, *Kluyveromyces lactis*; Mg, *Magnaporthe grisea*; Nc, *Neurospora crassa*; Oy, *Oculimacula yallundae*; Pa, *Podospora anserine*; Pm, *Penicillium marneffeii*; 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 $\beta$ Subunit Gene *PbGPB1* from *Pb01*

A G protein  $\beta$  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  $\beta$  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  $\beta$  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  $\beta$  subunits of *S. cerevisiae* (Ste4) and *S. pombe* (Git5) is further than the distance between PbGpb1 and the G  $\beta$  subunits of humans (e.g. Gnb1, Bnb2, Gnb4 and Gnb3). The percentage of identity to PbGpb1 is as follows:

<b>Protein: organism</b>	<b>% identity to PbGpb1</b>
AnSfaD: <i>A. nidulans</i>	93%
CpCpgb1: <i>Cryphonectria parasitica</i>	83%
MgMgb1: <i>Magnaporthe grisea</i>	81%
NcGnb1: <i>Neurospora crassa</i>	81%
UmBpp1: <i>Ustilago maydis</i>	72%
CnGpb1: <i>Cryptococcus neoformans</i>	70%
HumanGnb1: <i>Homo sapiens</i>	66%
HumanBnb1: <i>Homo sapiens</i>	64%
HumanGnb4: <i>Homo sapiens</i>	63%
HumanGnb3: <i>Homo sapiens</i>	62%
Ste4: <i>S. cerevisiae</i>	35%
Git5: <i>S. pombe</i>	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  AAAAGCCGAGGAAATCGGTAACCTGTCAACATGTCAAAAACATTTTTGGT
-1500  GGAGCATAGACACGGGATAGTTACTAATAGAAATGTTGTTTTATGAATAG
-1450  GCAAGGCACTGATGCTTCGTCATTGAGAAGTACAAATTTCCCGAAATCCC
-1400  AGCAAACCTCCAATGACCCAGCTCCATTGGTTTTTTTTTTTTTTTTTTTTT
-1350  GGTGGCCCTTCTGGAAACCGTCTCCAGAATGCTGGTGTGCGAGGAAGTCC
-1300  GTCCCCAAATGATTTGACAATTGCGTCTGGCTAGGTCCGCAAGGCGCC
-1250  TGAAGAAGTCTTCCATGGAAGTTTTTGTCTTCCACAAACACCCTGGCGA
-1200  CTCTTAGGCAAGCTGGTGTCTCTGACGTGCCAATAGGACAGTAAGTAATA
-1150  ATAAAGGGTGTTCCAATCATCTACAACCTATGTTTGTGCTGACACCGAGCA
-1100  CTGAGCACTGAGCAGTCAAGGAACGGGAGGAAAGAAATCAAAAATTGT
-1050  AACAAAAGGAGAATAGATAACCGTGTCAAGTCCCAAATAAGACGGGTGG
-1000  TTGGAATATTTCTGGTGTAGCCTTCTGTATCAATCGCTTTTCAGCCACA
-950   GCAGGGCAGATGCATGAACGCTGTTAATATTCAGACATAGGAAAAAACT
-900   TTGTATTTAACTTATCGGTGTCATTCCCTTGGTTTGTGTCAGCAGCGGATAT
-850   TTGTGGAGTAGAATACCGGGGATTAATAACAGCAGATATACCGACAGATT
-800   ATGGGCACAATACAACCCAAGTACATCCACGGGATATAGTGTATGGATAG
-750   ATACAGTTCTGTAGGCAAACGCACTCTGTACCGTGAGTGTAAATATTACTT
-700   ACCCTAGGTACCGCCAAACAGTTCCAAACCAGCACAAACCACTGTCTTC
-650   ATCAGCCTCAACCTTGAACCTCATCTCATCTTCATCTGCTCCTCTCAC
-600   TCACTCACCTTAATTTACCAAACCGCTCACACATCCCCAGACTGGAATT
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-500   TGCTGCTGGTCATCGTGGATTGCTTGTCTCCCAGTCTCTCTCTCACCC
-450   TGCTGCCAGCCATCCCTGAGCATATCCAGTGGTTGGTGTGGCCATTTGCC
-400   CTGTCTGTGCTGTGGTAGAGGGGACGACTCTTCTGTTTACTATATTTCAA
-350   GATCTGTACCTTCGCATCTGGTGTCTCCACCCTGCAACTGCTGGAGTC
-300   CCAAGTTCTCCACTCTATCTCATTAAACCATTATTCAGCCAACCACCCTC
-250   TCTGCCTCCTGTCCCAGGGCCCCAATAATAGTGTATTTGGTGAATCCC
-200   AGCTAGGTGCGTTTTCTAGTTCCCACATCATTTCTCCCAACCACAACCACA
-150   ACGGTTACCACAACCCTCCACTGCCTCCTCCCCGATCGACCATTGCGCA
-100   AATTTTGCTCTCCGTTAAACTACCACCCTTCTTCCTATCCATCCTCTTCC
-50    CATCCAGTTGACCACCCTCTCTCTATTCTTTGTGGTCTCGTTAATCTACC

      1  ATGGCGGCCGATTTGAGCGGCGAGCAAATGCAGGCGAAGATCACCGCTGC
      1  M A A D L S G E Q M Q A K I T A
     51  CCGTTCGTGAGGCAGAGGGTCTAAAGGATCGCATCAAGCGCAGGAAAGATG
    17  A R R E A E G L K D R I K R R K D
    101  AGCTTGCGGATACCTCTCgtaaggactgctgtctcttttactcttcaacc
    34  E L A D T S
    151  catctccacatatgttatcaccaccatggcatctatgaactcctctgaa
    201  tctctctttacacaactctcgccattgaaccagccaagtcataagagt
    251  tttctcactcgttggggctttctcgcacatgtccaccagtcattggetct
    301  atccccatcccagggttaaacccaggccattggttaattaactgacat
    351  aacataccttccctcatagTTCGCCAAGTCGCACAGAACAATACGGACCCC
     40  L R Q V A Q N N T D P
    401  CTTCCACGAATTGGAATGAGACCCGACGGAACCTAAAGGGCCACTTGGC
     51  L P R I G M R P R R N L K G H L
    451  CAAGATCTATGCGATGCACTGGTTCGACAGACCGCCGATCTGGTTTCTG
     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  GTGCACGCCATCCCCCTAAGATCGTTCATGGGTGATGACTTGCGCCTATGC
    101  V H A I P L R S S W V M T C A Y

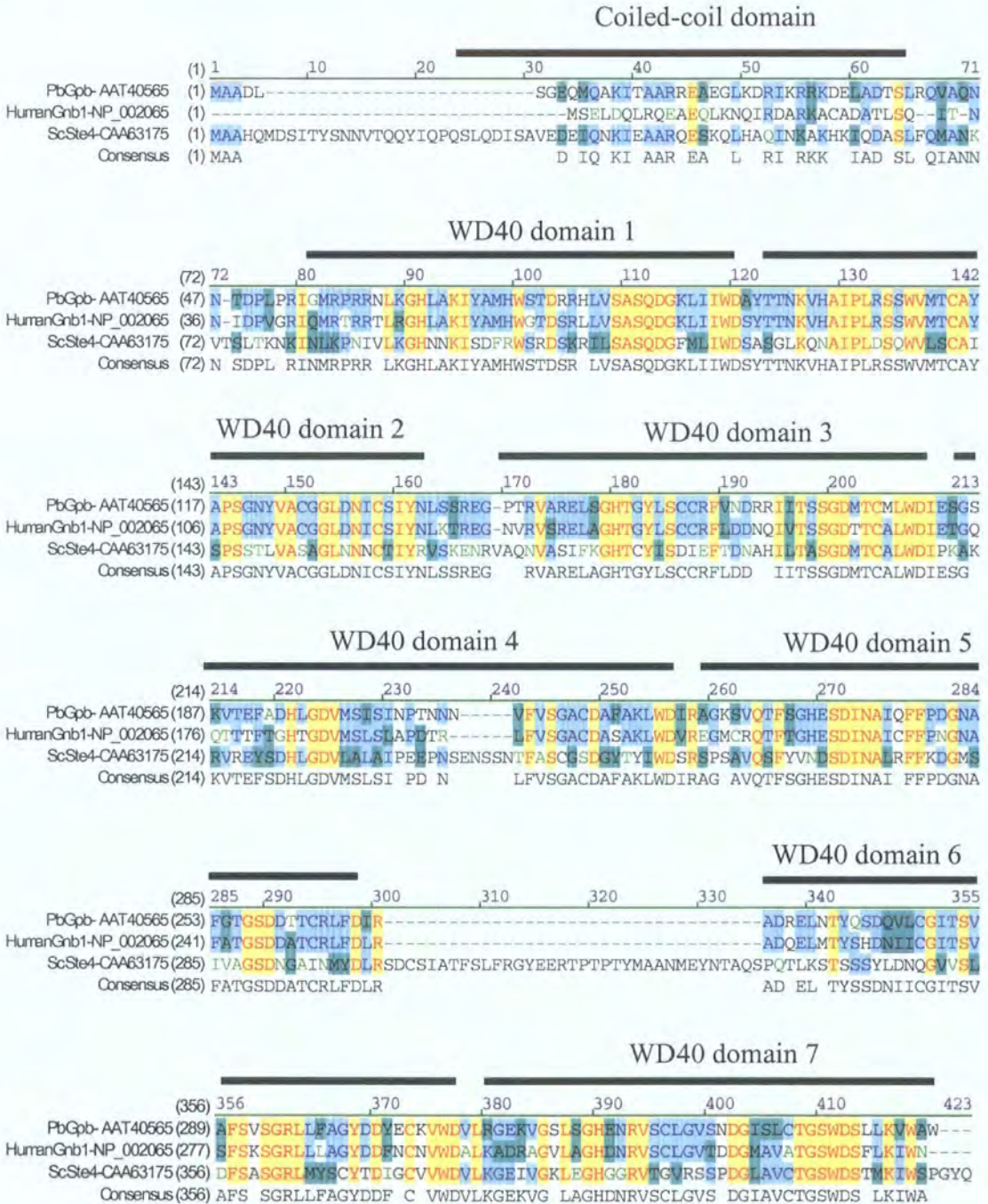
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601 CCGAGCGGTAACATATGTCGCCTGTGGTGGTCTAGACAACATCTGTTCCA  
117 A P S G N Y V A C G G L D N I C S  
651 TCTATAATCTTTCTCTCGAGAAGGTCCAACCCGTGTGCGACGAGAATTG  
134 I Y N L S S R E G P T R V A R E L  
701 TCCGGCCACACCGGTTACCTGTCATGTTGTCGTTTCGTAATGACCGTCG  
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 ATCAGCATAAACCCGACAAACAACGTTTTCGTCCTCCGGCGCATGCGA  
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 AATGCTTTTGGTACCGGATCGGACGACACCACCTGCCGTCTATTGATAT  
251 N A F G T G S D D T T C R L F D  
1051 CCGCGCGGATAGAGAACTTAATACATACCAGgtaaatattattaattcaaa  
267 I R A D R E L N T Y Q  
1101 cgctcatgacttaaattgtcaacataaaaagttcgttggataactgacat  
1151 gatcttttatattatacgcagAGTGATCAAGTACTTTGCCGTATTACTTC  
278 S D Q V L C G I T  
1201 CGTTGCGTTCAGTCTCTGGTTCGACTTTTGTGTTGCTGGCTACGATGATT  
287 S V A F S V S G R L L F A G Y D D  
1251 ATGAGTGCAAGgtaagtcaacctatttatttgetatcccttttgtgcgaa  
304 Y E C K  
1301 ccaactaattaatacgtaatcttttcaagGTCTGGGACGTTCTAAGAGG  
308 V W D V L R  
1351 AGAGAAGGTCCGTTCACTGAGTGGCCATGAAAATCGTGTGCTGAGCTGCCTGG  
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 gtgagcgttcaatgattagttcaattctacagtgaagttcccttgtgga  
1501 tatatggtttttttccaatcttcagCTCAAGGTCTGGGCATGGTAGACTT  
348 L K V W A W \*  
1551 GGTTTTGGCCAAATTATCGATCGTTGATGGTGGAACTGAACGAACACCCC  
1601 ACACCACCCGCCTTCAATTTGCAGTCTCGTACCACACACCCCTCCCTCC  
1651 CTCCCATCGACAAGGAAAGTTTTTCGCCTACAACATTCATATCCGTATA  
1701 TCCGTTTCTCTCCACACAAACACCATGATATCCTCGAGGAATCTGTCCA  
1751 TCTACAGGACAGGCATATATAATTGCAAGCATGCTTCTCAAAAATTTAAC  
1801 TACAGGGGACGGACTCGAGCTTCTCGTCTCTTCTCACAACAGCCTTCT  
1851 TACTCTGTAAGGCGACACAGAATAACATCAAACCTATGATGACATGAAG  
1901 GAAACAAACCTTGTTCCCTAATCCTCGCCATCCCTGCACACCTCTCTGAC  
1951 ACTATAACCCCTTCTTTTGTCCCTAAGTGTCTTAATTAGTGTCTTAAT  
2001 TTTCCGAACTAGAAGAAGATGAAAAGAGAGGAGTCAAGATCCTACCTTTT  
2051 ACTTTCTGCTCACAAAAGCTCACGAAACGAAAAGAAAGACACTAGGAGG  
2101 AATAAAAAATTGAGACTCTTGAGCTGCGAGATATCATTGGATAATAATC  
2151 TTTTGTATGGGATTTTCTTCAATTTTGTCTTCACTTCACTATATCCA  
2201 AGTACCTAATTTCTGGTTGCCACTTTTCCAGCTTTTGTGATGTTTTCTG  
2251 TCTTTTAAATGGCTCTCTGTTCTCCCCCTTACTGTTTACCTACACATAC  
2301 ACCGTTTTACTTTCTTTATGGNGCTGGTTTCCCTTATTTTCCCTCCCGTTA  
2351 ATTTTTTATACCTCGGGCTTGAAAGGATTTGTGAGCCTTATTATTTTCC  
2401 TTATAGTCTTTTGTCTCCGTGTTTAAACCCCATTTATCAGGCTATAAGT  
2451 TTCTCATTTTTCTTCCCTACTTTCTTTGCTGCGGGTTTCTCTCTCT  
2501 TTCTCTCAAAAATTTCTTTTGGGGATTTCTTCGTCGTTTCTTCCCTGT  
2551 TTGATTCCTACATTCCGAATTGCCCTAGGTGTGTATTACCTTTTTCCCT  
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2651 TCCTAGGTCTGTTCTTAACCCACGTTCTATTTCCATTATGTTTTCT  
2701 TATCTCC

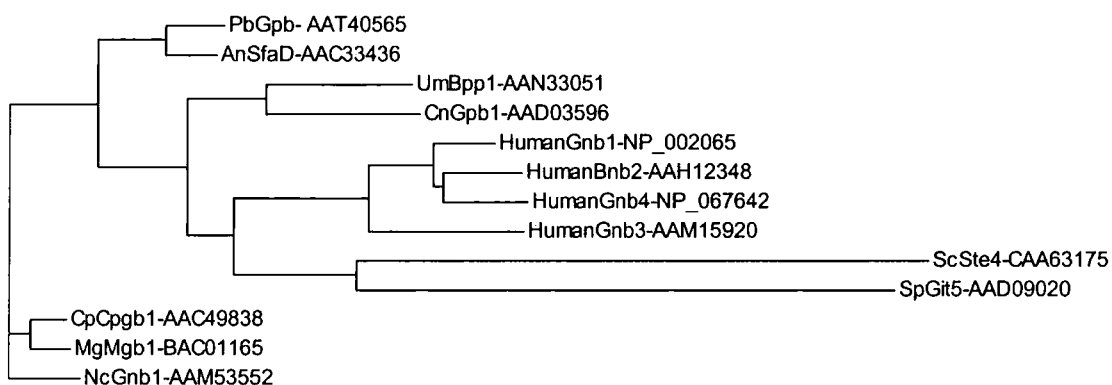
**Figure 3.8-2. Comparative alignment of PbGpb1 with a G protein  $\beta$  subunit of human being and the Ste4 protein of *S. cerevisiae*.**

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

G protein  $\beta$  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  $\beta$  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 synthesized; namely TPK-DF1, TPK-DF2, TPK-DR1 and TPK-DR2, corresponding to amino acid residues GTGSFGRVH, QVEHTNDE, YRDLKPEN and DVKSHPPWF, 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*.

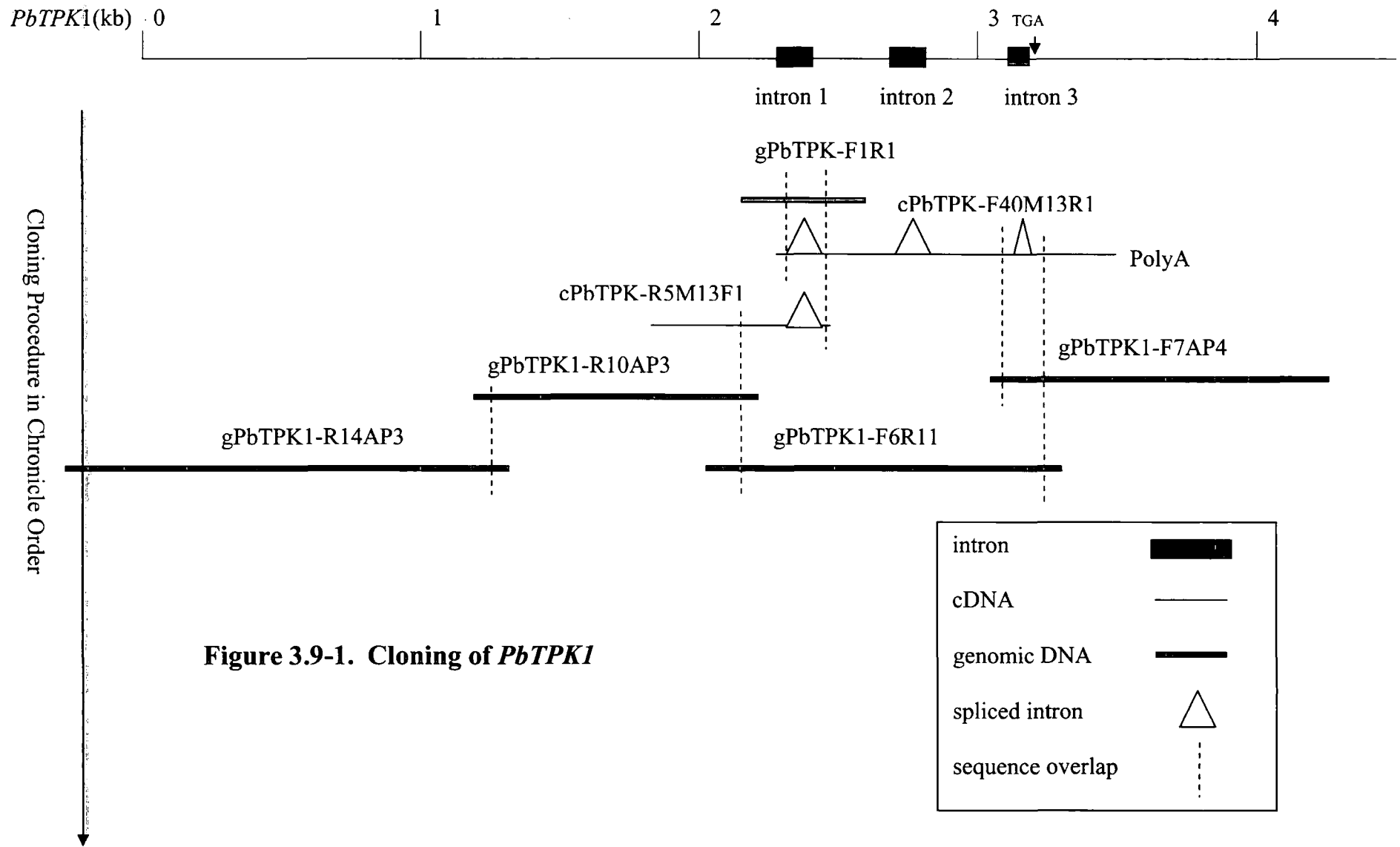
In order to check if there is a *TPK*-like gene in *P. brasiliensis*, 5 degenerate primers were designed based on the sequences of *TPK*-like proteins from *Aspergillus nidulans* and *Candida albicans*. These were 3 forward primers TPKL-DF1, TPKL-DF2 and TPKL-DF3, corresponding to amino acid sequence 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. cerevisiae* 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:

<b>Protein: organism</b>	<b>% identity to PbTpk1</b>
AnPkaA: <i>A. nidulans</i>	54%
NcPkaA: <i>N. crassa</i>	54%
MgCpkA: <i>M. grisea</i>	52%
ScTpk2: <i>S. cerevisiae</i>	43%
ScTpk3: <i>S. cerevisiae</i>	39%
ScTpk1: <i>S. cerevisiae</i>	39%
CaTpk3: <i>C. albicans</i>	40%
CaTpk2: <i>C. albicans</i>	42%
CnPka1: <i>C. neoformans</i>	42%
SpPka1: <i>S. pombe</i>	38%
UmUka1: <i>U. maydis</i>	28%
AnPkaB: <i>A. nidulans</i>	29%
NcPkaB: <i>N. crassa</i>	26%
MgPkaB: <i>M. grisea</i>	27%
ScSch9: <i>S. cerevisiae</i> (kinase-like protein)	19%
CaSch9: <i>C. albicans</i> (kinase-like protein)	19%
AnSchA: <i>A. nidulans</i> (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 CTCAAAGTGGAGGAGCCAATCGAATTCGAACTCGAGAATGGAACAAATGAG
1 Q T E E P I E F E L E N G T N E
51 ACGCTATCATCTGTACCGATCCAACGATCCAACAGTGACTCAGGTGCGCC
17 T L S S V P I Q R S N S D S G R
101 CATGGCTATCCCAATGAAAAGTCGACAAAAGTAGTAACAACAGCATGTTGG
33 P M A I P M K S R Q S S N N S M L
151 ATAGTCATGGCCATAAAGGGCTAAGTGAGGTTACAGATCCATTGTGGAAT
50 D S H G H K G L S E V T D P L W N
201 CATGAAGCCGTTTTgtgcgcttttgccttcttttcttttctactcattatcc
67 H E A V
251 ttttaattattggcaaaagcctatgcgcgctccttatcttactgaccttatta

301 cttctagTGATGTCCCTGGGGATGAATCTGAAATTGAAGTTTCGGTATAC
71 F D V L G D E S E I E V S V Y
351 GATCGATCGAACCACGAAGCGTCTTGGGTCATGTAAAGTTGCGTGTGAG
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 TTTTCAGAAAACAGACAAGAAGCAGTTTGGACCAAATGATTTCCAAATCCT
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 AGGACACCCAGAGAATCTACGCGATGAAAGTCCTTTCCAAAAGGTGATC
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 CCGGACTGCTATGACGAATTCTCCTTTTCATAGTTGGTCTCAAATTTTCTT
202 V R T A M T N S P F I V G L K F S
751 TTCAAACACCCACAGATTTGTACCTTGTACAGACTACATGTCTGGAGGG
218 F Q T P T D L Y L V T D Y M S G G
801 GAGCTGTTCTGGCATCTGCAAAAAGAAGGGAGATTTTCAGGAAGCTAGAGC
236 E L F W H L Q K E G R F Q E A R
851 GAAGTTTTACATCGCTGAGTTGATACTAGCTTTCAGCATCTACACGAAC
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 AATGGTCACATTGCGCTCTGCGATTTTGGTCTATCCAAGGCAAC
286 N G H I A L C D F G L S K A N

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**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.

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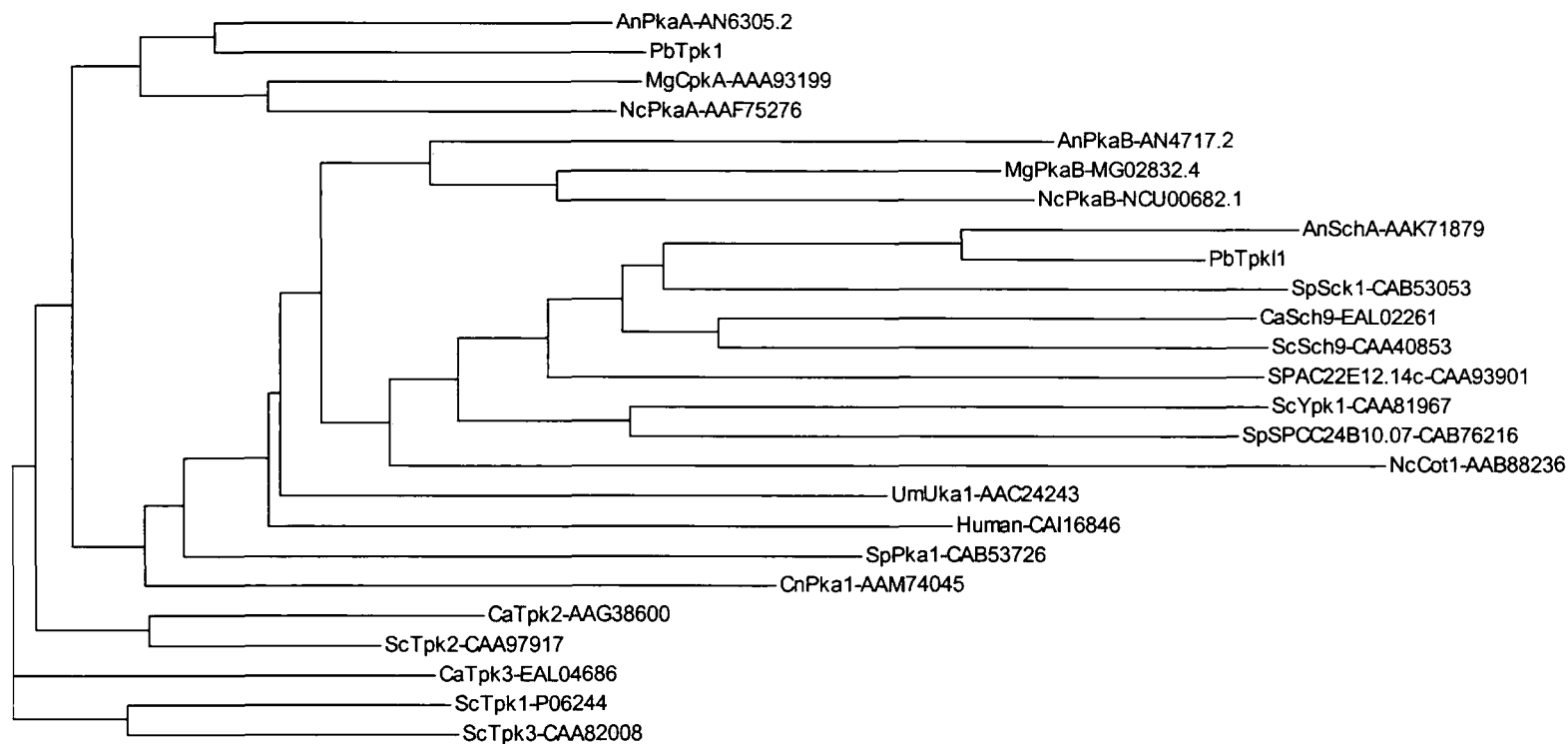
-2850 CAATCCTGGCATAACAAATAGAGAGCAAAAGGCACTAGAAAATTGGGGCTG
-2800 TGGGACAAGCTGCAGGACCAGCAGCTTCCAAAACCTGGCTTCAGAAAAGCT
-2750 GTATCCCCCTCAATCTCATCACATATACTGATGGCTCCAAAGACAGTCA
-2700 AGGAGTTTCAGGCGGAGGCTACTGTCTATACTGAGGAACAAATCAATCTC
-2650 TGGAGCTTGGTAAGCTGCCTCTGGGCCACACTGCTGAGGTCTTTGATGCT
-2600 GAAGTGTTCGAGCAGTTGCCAGTCTTGAGGCAGTGTACGGAATCCCTT
-2550 CTCTATGTTCCACAGCACATTATAGTGGCTCTAGACAATGAAGAGGCTG
-2500 CAATAAGACTCCACACAGACATGCCACACGAACATCTTTTCTCACCTTT
-2450 GCTAAGTTCAATGAACTCCACAGGTCTTGGACAAAACAGGGCCCAGGTCC
-2400 AGCCAGCCTGAGACGGGTCTATGTTTCGATGGTGCCCCAGCCATGCTGGGA
-2350 TTCCAGGCAATGATGCTGCAGATGCTGCGGCCAAGTCAGCCTGCACAATG
-2300 GAATGCACTCACCGCACTCTGACCCCTGGCACGGGCCAAATCCTATGCTAA
-2250 AGAACACTTCAATGCCTCACTGCAACAATACTGGAGCTCCAATAGCACAT
-2200 CACGATACAAGGACCTCAATATCCCAATGCCAAAAGGACCCTCCCCTGAG
-2150 CTCCTCTCCCCTGAAGGAACCTAGGCCACTTGCTGGCAGCTAGAACAGG
-2100 CCACAGTGACTTTGCAGCCTATCATCACAGATGGGCACATGACAATGCAC
-2050 TCCTCACCTGCTCTTGCGGCCGAGATAAGACCCCCGAGCACTTCTTCTTT
-2000 TGCTGGAAAAGGCTGGAACATTTGAATGAATTTAAAACCCCTGAAGCATGCTG
-1950 AGGACCAAAGGAAGCAATTTGACTGGCTTCTCAGCACAGTAAGAGGAGCTA
-1900 TGGCCTTCTCTACCTGGTGCACTAATACCCGCTTCTTCATCAACATCCAA
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-1600 GATACCCCTTAGGGAGGTGCTGTGGCCGCGACGGCAGACTATTCTTAGTCA
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-1000 GGTGAATAAAATTCACCAACTACTGTCTTCTGAAATAACCATATATCTAT
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-900 AAATATTATTGCGTTGCTGAGATGCCATTTGAAAATATCCAGAGCTGGTAG
-850 ACGAATTCATTGCGTAATAGAGTTTGTGGGCAGCTGGATTAACAACTG
-800 TGCTATATATAGGTCAGAACAAAAAGCATAGTGATGCATATAACCCATCT
-750 CTCTATCAATGTCTTCTCTCGGTTAAAGAGTGTGATACTAGGGCGAGA
-700 TATTGTTTATAGGTTATGATGTAAGTTTCGGGGTCCCCTCTGTTCTGGGCGG
-650 GACCACATACTCCGAGCAGGCACCTCATCCATAGTTATCTAATTCCCTTG
-600 TTATAACTAACTATCTAGTGGCGTTCGGGCTCAGCATCATCGACGTCTGC
-550 TTACTACAACGTCCCATTACTACCTCAACTACGGCCGTCATCTATTCCGA
-500 ACGAGAGTACCAACAAAACGTCAATCTTTGTCAAGTATTATAATTGCTCCC
-450 CAGGCACCAGAATCAACGAATCAACCCCTCTACCATCGCCAATTCCTTTA
-400 CCCTCCATTCCCCTGCCTCTCCGGTCCGTCCATTTCGTCGCCGTCTACGCA
-350 AATGCCTGCACTCGTTTGAACCTCACAGGTATCTGCTCCACCCTCCACAT
-300 TAAGCACTCCCCTCCGTACTTCGGATCCGTCGTTCGACTATGTATTAA
-250 TTATTGCCACAGACGGCGAAGCCAATAGGTCCCCCACAGATCCCTAACC
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-150 TTAGATATCGGGAGCGTACCAACGCAAAAGGCCGTCGATAGATCATTGGA  
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-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  
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51 S P L F L T T A A T A T A P A T  
201 AACAGCAGCACCAACAACGAACACCCTGCTCCAGCACCTGCGTTTACCT  
67 V T A A P T T N T L L Q H P A F T  
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84 S P A R D S S C K D P A S S P G T  
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167 A G D P S S H F K Q H 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  
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901 AAGGTGCTGAAGAAAGCGCAAGTGGTGAAGATGAAGCAGGTCGAGCATA  
301 K V L K K A Q V V K M K Q V E H  
951 GAATGATGAAAGAAGAATGCTGCAGCGGGTTAAACATCCATTCTAATTA  
317 T N D E R R M L Q R V K H P F L I  
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334 T L W G T F Q D V K N L Y M V M D  
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351 F V E G G E L F S L L R K S Q  
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1151 ttttgaactaattgatatcggttccctcagCGCTTCCCAAATCCTGTTGCA  
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 ATGGACATTTGAAGATTACAGATTTTGGATTTCGGAAGGAAGTCAAGGAT  
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  
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1451 cccccccccccccccctcctttttatttttatttttattttttgtattg  
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450 W W S L G I L I F E M L C G F T  
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1701 TCCCAACTCATTACTCCCGATCTCACAGTACGCCTGGGCAACTTGCACGG  
500 S Q L I T P D L T V R L G N L H  
1751 CGGCAGTAAGGATGTTAAGGATCATCCGTGGTTTTCGGGAGGTGACGTGGG  
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  
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550 G G Q G D A S Q F D R Y P E E T  
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566 E P Y G G S G D D  
1951 ctcggttgcttcaacgcagcaagctttactaatgatggttatagATATGGCG  
575 P Y G  
2001 ATTATTTTCGTGGACTTTTGAATGGGGATAATTTTGTGAAGACCCGTCGAC  
578 D Y F V D F \*  
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2201 CGGGAAACGATGTTTTATTCAATTCTCTCTCTTGAAGATAAATGTATGCA  
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2351 CTCATTTTCATCATTTAACTCTATCTTGCTCCATGGGACGGTTATACCTG  
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2551 TGTTTTAATATTATTGGACTTTTGTGGGGATGACATGTTCTTCTCAGAG  
2601 TATCTTTTTGGTCAATGGAATGGGCTTGCTATGGCGATCCGTGACATAAA  
2651 ATCAGTGAGTTGATGTAATGGGAAATGAAAATGCTGTGAAAAGAGAAGAT  
2701 AAACGTTTTGTTTCCCTATGGTCATGTATTTTCATATCTTCTACCGTTTTT  
2751 GTCTCTCTTTTCTCGTTCATCGTACGTCGCTTATTCCTGGATCCTACATA  
2801 GTCGTCGTGAATAGATTTTATAGCCCGACTTAATAGTCAGACTTAAAATA  
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2901 ATCCTAACTTTTGTTCATGGATGATAGAAACCATTCCTTCGTGTGATTCAC  
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3001 TGGAAAAGCATAAACTATAAATACAGGGAGATAAAAACAAGACCACCAG  
3051 TAGGAGGTTATAGGGAGCAGGTGCGTGCAAAAAGGGGGGGAAAAGCGAG  
3101 GAGATATATAAA

**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. cerevisiae*; 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. marneffeii* 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.

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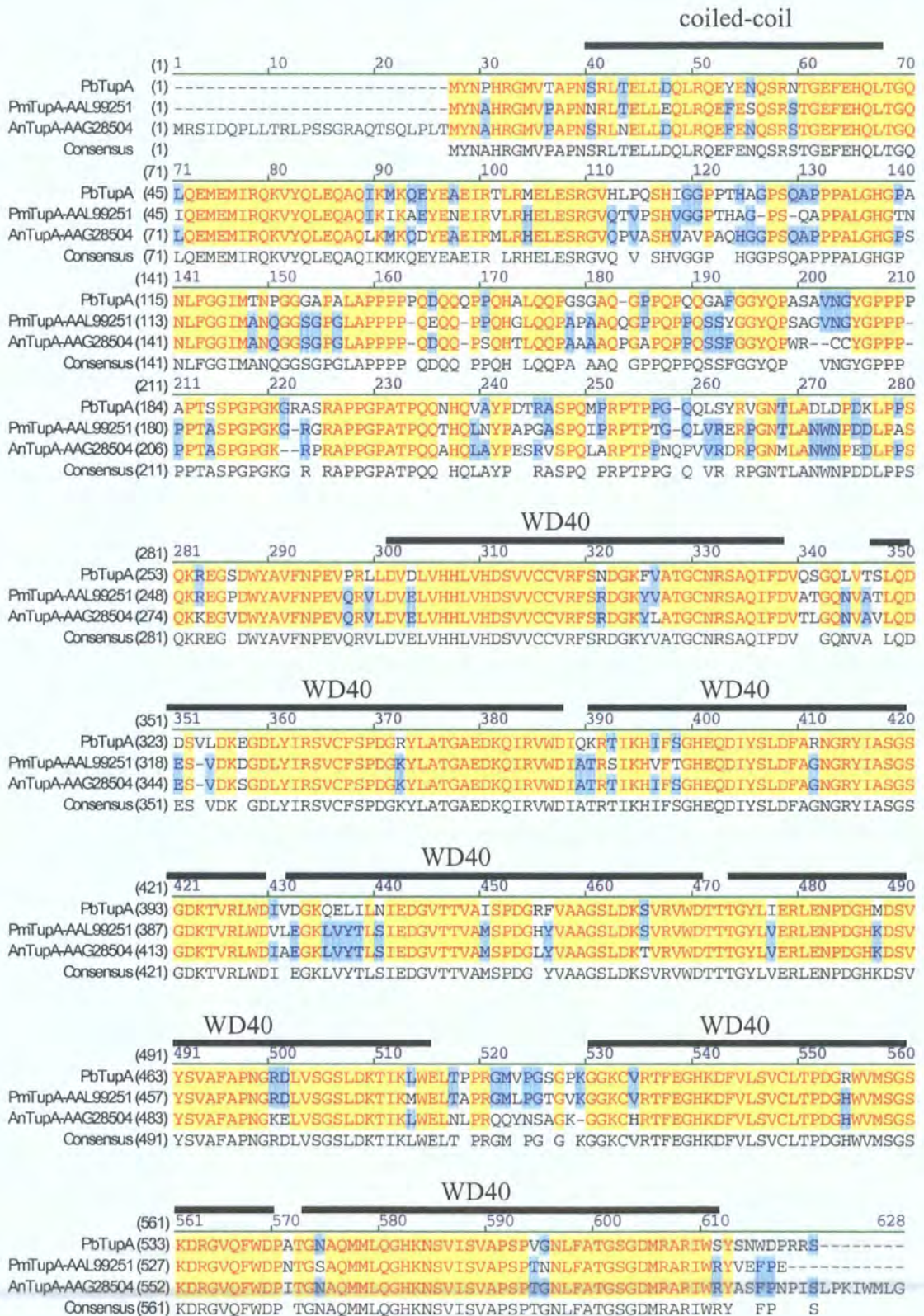
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-50 AACATAACTTCTTACCTTAGGCCAGCCTCGCCCTCAAGGCTTGTGCCAAC

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1 M Y N P H R G M V T A P N S R L
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17 T E L L D Q L R Q E Y E N Q S R N
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34 T G E F E H Q
151 ctttcttctcttcttcttcttcttcttcttcttcttataataccctcccaacct
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41 L T G Q L Q E M E M I R Q
401 AAAGTGTACCAGCTGGAGCAGGCCAGATAAAAAATGAAGCAAGAgtagct
54 K V Y Q L E Q A Q I K M K Q
451 cttcggctcttatttataatcctcataaatgatctcgagtgagagtgttt
501 gctaactgtctctgcacttgccttcaagATACGAGGCTGAAATCCGCACT
68 E Y E A E I R T
551 CTTCGAATGGAACCTCGAATCTCGTGGCGTGCATCTCCCCAGTCTCACAT
75 L R M E L E S R G V H L P Q S H
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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
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125 G G A P A L A P P P P P Q D Q Q
751 ACCGCCCCAGCACGCTCTTCAGCAACCCGGTTCGGCGCCCAAGGCCCTC
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
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273 D V D L V H H L V H D S V V C C V
1251 AGGTTAGCAATGATGGGAAAATTTGTTGCTACGGGCTGCAATCGCTCTGC
290 R F S N D G K F V A T G C N R S
1301 GCAGATCTTTGACGTTTACAGAGTGGACAGCTTGTACCTCTTTGCAAGATG
306 A Q I F D V Q S G Q L V T S L Q D

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1351 ATTCTGTTCTTGATAAGGAAGGGGACCTTTATATCCGTAGCGTCTGCTTT  
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1401 AGTCCAGATGGGAGATACCTTGTACTGGTGCAGAAGATAAACAGATTAG  
340 S P D G R Y L A T G A E D K Q I  
1451 **Agtaaggacatgcccttgttctttttcttttctgctatttatattgtt**  
356 R  
1501 **ccattccaccaactaacctccacctacag**GTCTGGGATATCCAAAAACGC  
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 CTTTCGCCCCGAATGGCCGCTACATTGCCTCCGGCTCCGGCGACAAAACCG  
379 D F A R N G R Y I A S G S G D K T  
1651 TCCGCCTTTGGGACATCGTCGACGGCAAGCAGGAAC TAATCCTCAACATC  
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 TCTGTGGCGTTTGCGCCGAATGGGAGGGATCTTGTGTGGGCAGTTTGGGA  
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 **gtgggtttcccctcttttgtgtcaattcaagttgatgaaggctgtaact**  
2051 **gacatatctatgtttcttcgcag**GATTTTGTCTTAGTGTCTGCTTGACA  
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 ATTCAG**gttcggttattcgtttttattctttgtttctgcgggccccctct**  
556 N S  
2251 **tttgtctctcaagctaaccatattctccttcggttag**TTATCTCCGTGCGA  
558 V I S V A  
2301 CCCAGCCCAGTCGGCAACCTGTTTGCAGCCGGAAGCGGAGACATGCGTGC  
563 P S P V G N L F A T G S G D M R  
2351 TAGAATCTGGAG**gttagtttcaaccctcaacgtttgatatcatatatttg**  
579 A R I W  
2401 **cgtttctgttcatcctcgttatcaccgcccgtcatcatataattcgcgctc**  
2451 **ccttgccccttctaccatagtttctctttgtatccactcttattttattct**  
2501 **ccttcatgtcccagtataactaacgtctacaaaacctactctcgacatgg**  
2551 **gatttacag**CTATTCCAATTGGGATCCCAGAAGGTCATAGGTTATGTTA  
583 S Y S N W D P R R S \*  
2601 ATTCACAAGCGGACGCAGGCACGCTCACGCATATGGCAGA ACTTGCAAAA  
2651 CATGCAGTTGGGCATATATTTAAATATGCGCAACACGTA ACTTTGCCAGA  
2701 GGAAGGAAATAAAGGAGGAAAACGGGCCAAGGGAAAGGGAAAGGCGTTCT  
2751 GTGGCTACTACATTTACCGGCTCTTGCATTGATTGG

**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
<b>Constructions as templates</b>		
pYES6-cPbAC-F26R34-1-14	pYES6/CT (Invitrogen)	See Figure 3.5-1; The insert is the -21 bp <i>PbCYR1</i> 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 <i>PbCYR1</i> 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 <i>PbCYR1</i> 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 <i>Pb01</i> mRNA. The insert is the full-length <i>PbGPA1</i> 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 <i>Pb01</i> mRNA. The insert is the full-length <i>PbGPA2</i> 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- <i>Pb01</i> cDNA library. The insert is the full-length <i>PbGPA3</i> cDNA.
pGEM-TE-PbACTIN-GADF1R1-12(SP6>T7)	pGEM T Easy (Promega)	The insert is RT-PCR product amplified with PbActinGADF1 and PbActinGADR1 from <i>Pb01</i> mRNA. The insert is the full-length <i>PbACTIN</i> cDNA.
pGEMTE-ScGPR1-F1R1-17	pGEM T Easy (Promega)	The insert is the full length <i>ScGPR1</i> which was cloned by recombination of fidelity PCR products. The sequence of <i>ScGPR1</i> in this clone spans from the position of primer ScGPR1-F1 to that of ScGPR1-R1.
<b>Constructions for Y2H</b>		
pGAD- <i>PbCYR1</i> <sup>1-678</sup>	pGADT7 (Clontech)	<i>PbCYR1</i> <sup>1-678</sup> fragment from pGBK- <i>PbCYR1</i> <sup>1-678</sup> was digested with NdeI/BamHI and cloned into pGADT7 (NdeI/BamHI). This insert corresponds to the RA domain of PbCyr1.
pGAD- <i>PbCYR1</i> <sup>600-1316</sup>	pGADT7 (Clontech)	<i>PbCYR1</i> <sup>600-1316</sup> fragment from pGBK- <i>PbCYR1</i> <sup>600-1316</sup> was digested with NdeI/BamHI and cloned into pGADT7 (NdeI/BamHI). This insert corresponds to the LRR domains of PbCyr1.

pGAD- <i>PbCYR1</i> <sup>1302-1876</sup>	pGADT7 (Clontech)	<i>PbCYR1</i> 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- <i>PbCYR1</i> <sup>1648-2100</sup>	pGADT7 (Clontech)	<i>PbCYR1</i> 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- <i>PbGPA1</i>	pGADT7 (Clontech)	Full-length <i>PbGPA1</i> insert from pGBK- <i>PbGPA1</i> was digested with NdeI and cloned into pGADT7 (NdeI).
pGAD- <i>PbGPA2</i>	pGADT7 (Clontech)	Full-length <i>PbGPA2</i> insert from pGBK- <i>PbGPA2</i> was digested with NdeI/SalI and cloned into pGADT7 (NdeI/XhoI).
pGAD- <i>PbGPA3</i>	pGADT7 (Clontech)	Full-length <i>PbGPA3</i> insert from pGBK- <i>PbGPA3</i> (NdeI/BamHI) was cloned into pGADT7 (NdeI/BamHI).
pGAD- <i>PbGPB1</i>	pGADT7 (Clontech)	Full-length <i>PbGPB1</i> cDNA (NdeI/BamHI) was cloned into the vector pGADT7 (NdeI/BamHI); This construction was made by Dr G. Chen in our lab.
pGAD- <i>PbRAS1</i>	pGADT7 (Clontech)	Full-length <i>PbRAS1</i> insert from pGBK- <i>PbRAS1</i> (NdeI/EcoRI) was cloned into pGADT7 (NdeI/EcoRI).
pGAD- <i>PbRAS1</i> <sup>1-83</sup>	pGADT7 (Clontech)	PCR product amplified with 5'BD Screening and PbRAS-R1 as primers, and pGBK- <i>PbRAS1</i> as template was digested with NdeI/EcoRI and cloned into pGADT7 (NdeI/EcoRI).
pGAD- <i>PbRAS1</i> <sup>26-238</sup>	pGADT7 (Clontech)	PCR product amplified with 3'BD Screening and PbRAS-F1 as primers, and pGBK- <i>PbRAS1</i> as template was digested with NdeI/EcoRI and cloned into pGADT7 (NdeI/EcoRI).
pGAD- <i>PbACTIN</i>	pGADT7 (Clontech)	Full-length <i>PbACTIN</i> insert (NdeI/EcoRI) from pGEMTE- <i>PbACTIN</i> -GADF1R1-12 was cloned into pGADT7 (NdeI/EcoRI).
pGAD- <i>PbACTIN</i> <sup>1-314</sup>	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- <i>ScGPR1</i> <sup>679-961</sup> -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 <i>ScGPR1</i> .
pGAD- <i>ScGPR1</i> <sup>274-621</sup> -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 <i>ScGPR1</i> .
pGAD- <i>ScGPR1</i> <sup>1-961</sup> -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 <i>ScGPR1</i> .
pGADT7-T	pGADT7 (Clontech)	From Clontech
pGAD- <i>P53</i> <sup>72-390</sup>	pGADT7 (Clontech)	P53 insert from pGBKT7-P53 (NdeI/BamHI digested), was cloned into pGADT7 (NdeI/BamHI).
pGAD- <i>lam</i> <sup>66-230</sup>	pGADT7 (Clontech)	Lam insert from pGBKT7-Lam (NdeI/BamHI digested) was cloned into pGADT7 (NdeI/BamHI).
pGBK- <i>PbCYR1</i> <sup>1-678</sup>	pGBKT7 (Clontech)	<i>PbCYR1</i> 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- <i>PbCYR1</i> <sup>600-1316</sup>	pGBKT7 (Clontech)	<i>PbCYR1</i> 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- <i>PbCYR1</i> <sup>1302-1876</sup>	pGBKT7 (Clontech)	<i>PbCYR1</i> 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- <i>PbCYR1</i> <sup>1648-2100</sup>	pGBKT7 (Clontech)	<i>PbCYR1</i> 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- <i>PbGPA1</i>	pGBKT7 (Clontech)	Full-length cDNA of <i>PbGPA1</i> from pGEMTE-cPbGPA1-ExGPAF1pYER3-9 was digested with NdeI and cloned into pGBKT7 (NdeI).
pGBK- <i>PbGPA2</i>	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- <i>PbGPA3</i>	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- <i>PbGPB1</i>	pGBKT7 (Clontech)	Full-length <i>PbGPB1</i> 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- <i>PbRAS1</i>	pGBKT7 (Clontech)	Full-length <i>PbRAS1</i> 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- <i>ScGPR1</i> <sup>1-961</sup> -F3R1	pGBKT7	Insert (BamHI digested) from pGAD-

	(Clontech)	<i>ScGPR1</i> <sup>1-961</sup> -F3R1 was cloned into pGBKT7 (BamHI). This insert is the full-length <i>ScGPR1</i> .
pGBK- <i>ScGPA2</i> -F1R1	pGBKT7 (Clontech)	PCR product obtained with <i>ScGPA2</i> -F1 and <i>ScGPA2</i> -R1 as primers and <i>S. cerevisiae</i> DNA as template was digested with BamHI and cloned into pGBKT7 (BamHI); This insert is the full-length <i>ScGPA2</i> .

### 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*<sup>679-961</sup>-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 *NdeI/Sall*, and ligated into pGADT7 (*NdeI/XhoI*). The ligation product was transformed into XL-10 Gold Ultracompetent Cell (Stratagene), generating 4 X 10<sup>4</sup> 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 *NdeI/EcoRI*, and ligated into pGADT7 (*NdeI/EcoRI*). The ligation product was transformed into XL-10 Gold Ultracompetent Cell, generating  $4 \times 10^4$  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  $\mu\text{g}/\mu\text{l}$  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 *NdeI/EcoRI*, and ligated into pGADT7 (*NdeI/EcoRI*). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating  $4 \times 10^4$  transformants. 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  $\mu\text{g}/\mu\text{l}$  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 *NdeI/BamHI*, and ligated into pGADT7 (*NdeI/BamHI*). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating  $4 \times 10^4$  transformants. 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 undertaken using 5'BD Screening and 3'BD Screening as primers, and pGBKT7-*PbRAS* as template. The PCR products were pooled, cleaned, digested with *NdeI/EcoRI*, and ligated into pGADT7 (*NdeI/EcoRI*). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 4 X 10<sup>4</sup> transformants. 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 undertaken 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 (*NdeI/EcoRI*). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 2 X 10<sup>4</sup> transformants. 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*<sup>679-961</sup>-RM-Lib, random mutagenesis PCR was undertaken using 5'AD Screening and 3'AD Screening as primers, and pGAD-*ScGPR1*<sup>679-961</sup>-F5R1 (C-terminal cytoplasmic domain) as template. PCR product was cleaned, digested with *NdeI/XhoI*, and ligated into pGADT7 (*NdeI/XhoI*). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 2 X 10<sup>4</sup> transformants. The plasmid library pGAD-*ScGPR1*<sup>679-961</sup>-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 were cleaned, digested with *NdeI/BamHI*, and ligated into pGADT7 (*NdeI/BamHI*). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 4 X 10<sup>4</sup> transformants. The plasmid library pGAD-*P53*-RM-Lib was obtained with Qiagen's plasmid 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 *NdeI/BamHI*, and ligated into pGADT7 (*NdeI/BamHI*). The ligation was transformed into XL-10 Gold Ultracompetent Cell, generating 4 X 10<sup>4</sup> transformants. The plasmid library pGAD-*lam*-RM-Lib was obtained with Qiagen's plasmid 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.

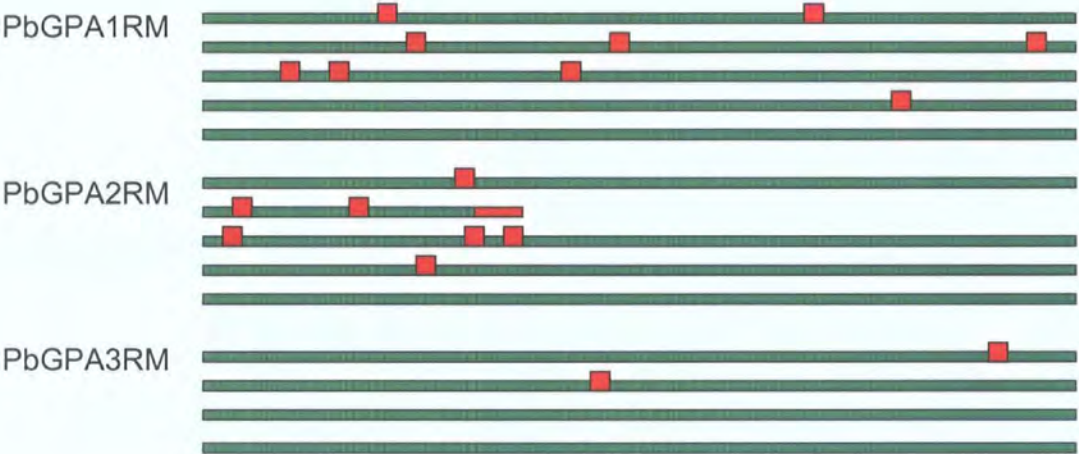
**Table 3.12. Random Mutagenesis Libraries.**

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

pGAD- <i>ScGPR1</i> <sup>679-961</sup> -RM-Lib	2X10 <sup>4</sup>	0.25 µg/µl
pGAD- <i>P53</i> -RM-Lib	4X10 <sup>4</sup>	0.4 µg/µl
pGAD- <i>lam</i> -RM-Lib	4X10 <sup>4</sup>	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.



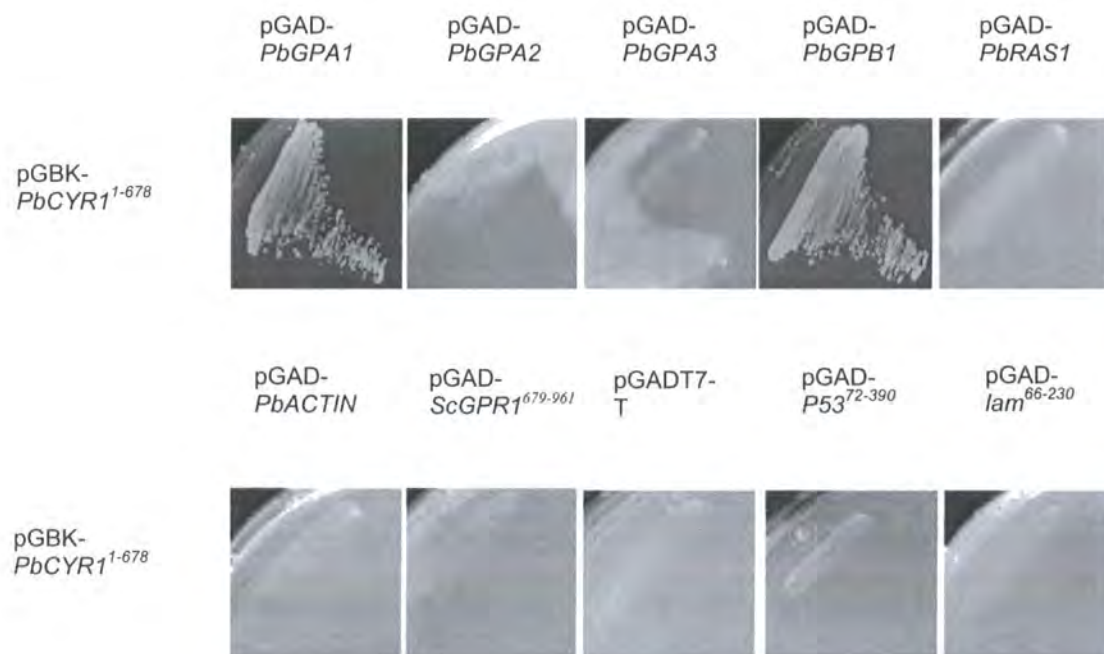
Random Mutagenesis Libraries	Mutations (5 clones tested for each library)
pGAD-PbGPA1-RM-Lib	E238D; Q98K, G202D, R348G; S47P, E65G, L194H; No mutation at amino acid level; E291V
pGAD-PbGPA2-RM-Lib	D119V; K14E, H61Q, mis-sense mutations from 86-97, Δ98-359; No mutation; D10E, P116S, E122K; L110P
pGAD-PbGPA3-RM-Lib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

### 3.13 Full-Length PbGpa1 and PbGpb1 Interact with PbCyr<sup>1-678</sup> in the Yeast-Two-Hybrid System.

As indicated in Table 3.11, segments *PbCYR1*<sup>1-678</sup>, *PbCYR1*<sup>600-1316</sup>, *PbCYR1*<sup>1302-1876</sup> and *PbCYR1*<sup>1648-2100</sup> 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*<sup>1-678</sup> because cotransformants with these 2 combinations of bait and prey construction grew well in SD/-Ade/-His/-Leu/-Try, while cotransformants 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. cerevisiae* *GPR1* C-terminal cytoplasmic domain were cloned into pGADT7 to make constructions pGAD-*PbACTIN*, and pGAD-*ScGPR1*<sup>679-961</sup>-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*<sup>72-390</sup> and pGAD-*lam*<sup>66-230</sup>. 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*<sup>1-678</sup> (Figure 3.13). Therefore, the following conclusion was drawn: full-length PbGpa1 and PbGpb1 interact with the first third of *Pb* adenylate cyclase PbCyr1<sup>1-678</sup> (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<sup>1-678</sup>.** 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*<sup>1-678</sup>, while full-length pGAD-*PbGPA2*, pGAD-*PbGPA3*, pGAD-*PbRAS1*, and pGAD-*PbACTIN* can not directly interact with pGBK-*PbCYR1*<sup>1-678</sup>.



	pGBK- <i>PbCYR1</i> <sup>1-678</sup>	pGBK- <i>PbCYR1</i> <sup>600-1316</sup>	pGBK- <i>PbCYR1</i> <sup>1302-1876</sup>	pGBK- <i>PbCYR1</i> <sup>1648-2100</sup>
pGAD- <i>PbGPA1</i>	+	-	-	-
pGAD- <i>PbGPA2</i>	-	-	-	-
pGAD- <i>PbGPA3</i>	-	-	-	-
pGAD- <i>PbGPB1</i>	+	-	-	-
pGAD- <i>PbRAS1</i>	-	-	-	-
pGAD- <i>PbACTIN</i>	-	Not tested	Not tested	Not tested
pGAD- <i>ScGPR1</i> <sup>679-961</sup>	-	Not tested	Not tested	Not tested
pGADT7-T	-	Not tested	Not tested	Not tested
pGAD- <i>P53</i> <sup>72-390</sup>	-	Not tested	Not tested	Not tested
pGAD- <i>lam</i> <sup>66-230</sup>	-	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<sup>1-678</sup>**

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 from *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*<sup>1-678</sup>; pGBK-*PbCYR1*<sup>600-1316</sup>;

pGBK-*PbCYR1*<sup>1302-1876</sup> and pGBK-*PbCYR1*<sup>1648-2100</sup> 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- <i>PbCYR1</i> <sup>1-678</sup>	pGBK- <i>PbCYR1</i> <sup>600-1316</sup>	pGBK- <i>PbCYR1</i> <sup>1302-1876</sup>	pGBK- <i>PbCYR1</i> <sup>1648-2100</sup>
pGAD- <i>PbGPA1</i> -RM-Lib	≈ 200	0	0	0
pGAD- <i>PbGPA2</i> -RM-Lib	55	0	0	0
pGAD- <i>PbGPA3</i> -RM-Lib	8	0	0	0
pGAD- <i>PbGPB1</i> -RM-Lib	≈ 100	0	0	0
pGAD- <i>PbRAS1</i> -RM-Lib	3	0	0	0

It is interesting to observe that all positive clones arose from screening with pGBK-*PbCYR1*<sup>1-678</sup>, 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*<sup>1-678</sup> (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<sup>5</sup>-10<sup>6</sup> 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<sup>1-678</sup>. 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*<sup>1-678</sup>.** Note: clones were selected randomly before sequencing analysis.

RM Libraries	Sequences of positive clones after screening with pGBK- <i>PbCYR1</i> <sup>1-678</sup>
pGBK- <i>PbGPA1</i> -RM-Lib	L91P, F199T. Δ211-353, K54N. Δ209-353, E65D, I107T. Δ160-353, L38M. Δ157-353. Δ155-353. Δ145-353. Δ139-353.
pGBK- <i>PbGPB1</i> -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- <i>PbGPA2</i> -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- <i>PbGPA3</i> -RM-Lib	V76L. Δ214-356. Δ198-356. Δ192-356, S30T. Δ189-356, V127A. Δ175-356, S7Y. Δ164-356. Δ161-356.
pGBK- <i>PbRASI</i> -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<sup>1-678</sup>. 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<sup>1-138</sup>, and PbGpb1<sup>1-179</sup> 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 ( $\Delta$ 184-359,  $\Delta$ 174-359,  $\Delta$ 103-359) 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.  $\Delta$ 214-356;  $\Delta$ 198-356;  $\Delta$ 164-356; and  $\Delta$ 161-356).

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 ( $\Delta$ 1-25+L107I) and the other, a C-terminal truncation with a stretch of mutations caused by a frame-shift ( $\Delta$ 149-238+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*<sup>1-83</sup> and pGAD-*PbRAS1*<sup>26-238</sup> 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*<sup>1-83</sup> and pGAD-*PbRAS1*<sup>26-238</sup> can interact with *PbCYR1*<sup>1-678</sup>, confirming that truncations without any additional mutation lead to interaction with *PbCYR1*<sup>1-678</sup>.

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*<sup>1-678</sup>. 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*<sup>1-678</sup>. 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.

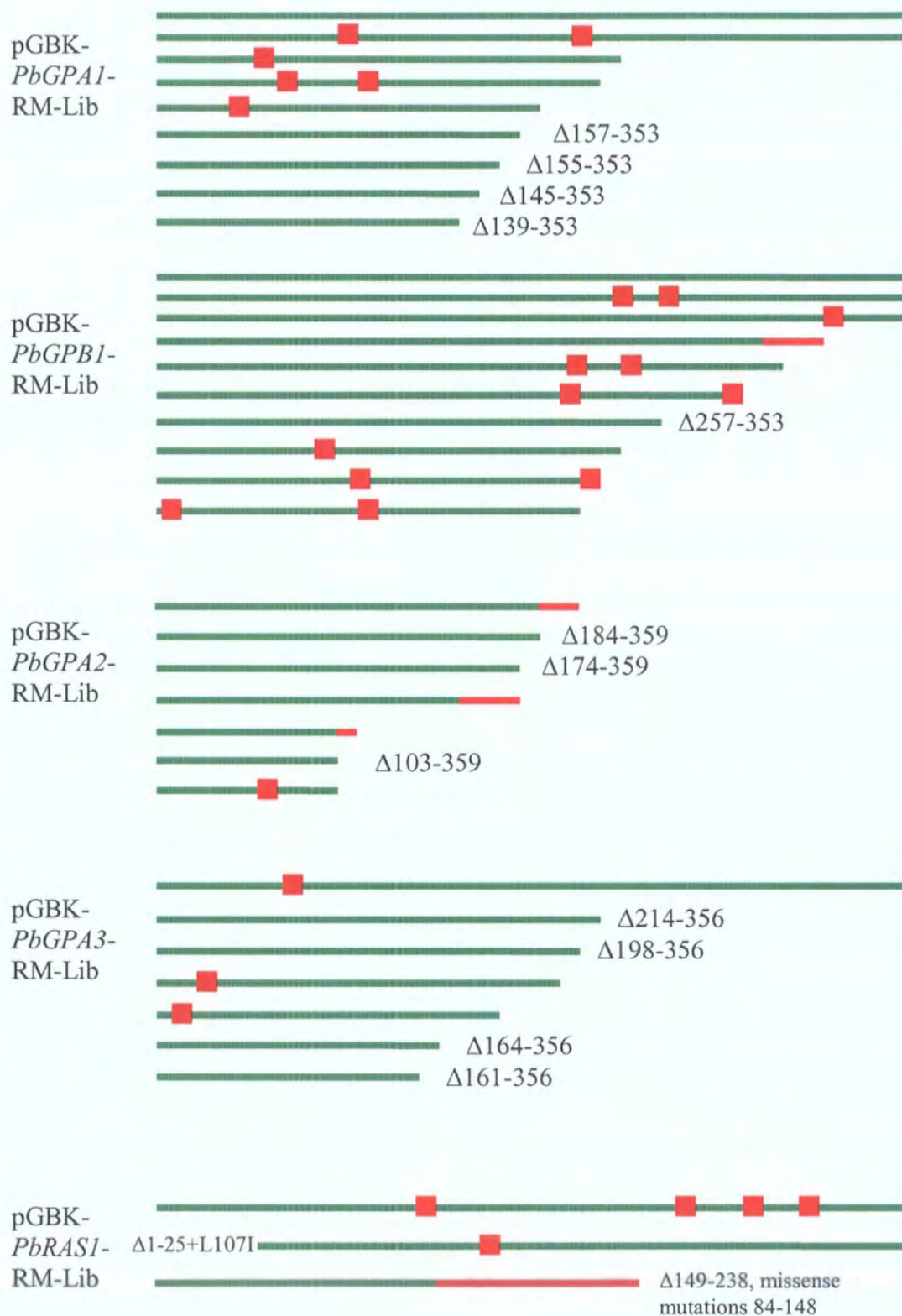
RM librariy	Sequencing results of 4 positive clones after screening pGAD- <i>PbACTIN</i> -RM-Lib
pGAD- <i>PbACTIN</i> -RM-Lib	Δ325-375, D244V, and missense mutations 315-324. Δ327-375, I10N, Q59H, V76A, R206H, and K326R. Δ336-375, R116P, L189F, missense mutations 316-335.

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*<sup>1-314</sup> was made as described in Table 3.11. Sequencing confirmed that the construction pGAD-*PbACTIN*<sup>1-314</sup> 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*<sup>1-678</sup>.

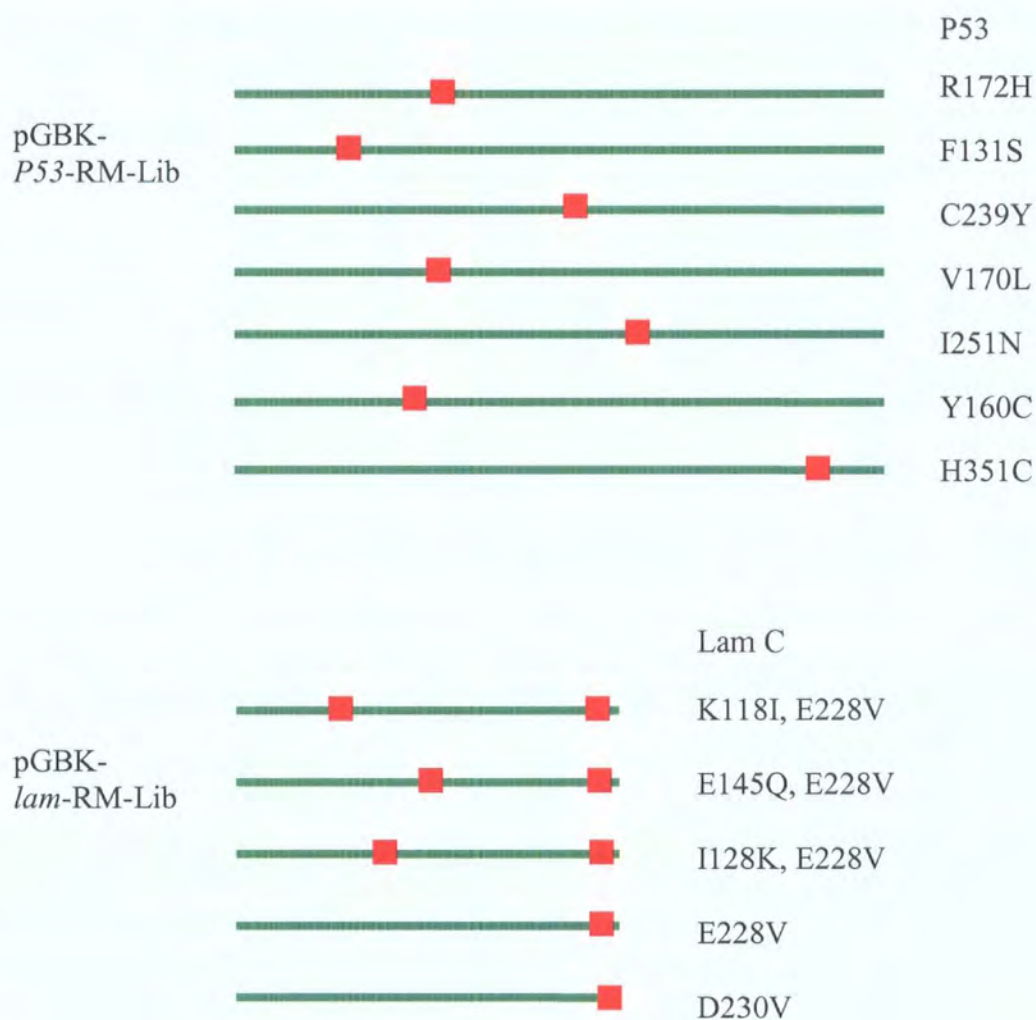
To exclude the possibility of false positives, 3 negative control RM libraries were made by recruiting 3 genes, i.e. *ScGPR1*<sup>679-961</sup> (C-terminal cytoplasmic domain, functional as indicated by interacting with *ScGPA2*), P53 and Lamin C, because they do not interact with *PbCYR1*<sup>1-678</sup> (Table 3.12 and Figure 3.13). No positive clones were obtained after screening RM library pGAD-*ScGPR1*<sup>679-961</sup>-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*<sup>1-678</sup> (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*<sup>1-678</sup>. 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*<sup>1-678</sup>. 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*<sup>1-678</sup> as shown in Figure 3.14-3.

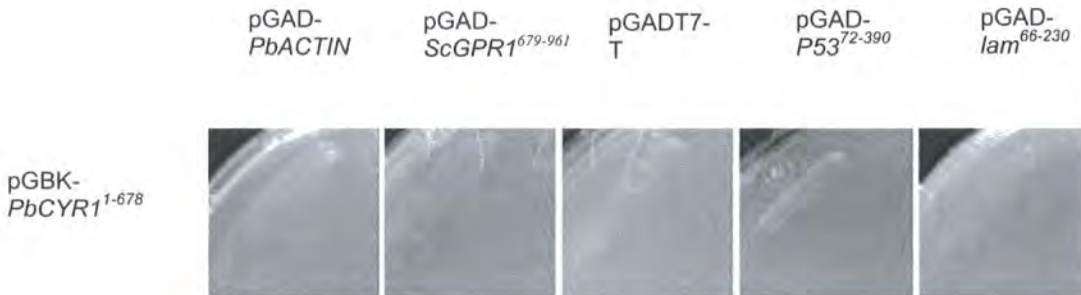
**Figure 3.14-1. Sequencing results of positive clones obtained from screening mutagenesis libraries with pGBK-*PbCYR1*<sup>1-678</sup>.** This is the schematic diagram of the table 3.14-2. Clones were randomly selected before sequencing analysis. Genes were denoted by green lines. Mutations are indicated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



**Figure 3.14-2. Sequencing results of positive clones obtained from screening negative control mutagenesis libraries with pGBK-*PbCYRI*<sup>1-678</sup>.** 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*<sup>679-961</sup>-RM-Lib.



**Figure 3.14-3. Truncated versions of PbGpa2, PbGpa3, PbRas1 and PbActin interact with PbCyr1<sup>1-678</sup>.** 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*<sup>1-102</sup>, pGAD-*PbGPA3*<sup>1-160</sup>, pGAD-*PbRAS1*<sup>1-83</sup>, pGAD-*PbRAS1*<sup>26-238</sup> and pGAD-*PbACTIN*<sup>1-314</sup> can interact with pGBK-*PbCYR1*<sup>1-678</sup>, while controls pGAD-*PbACTIN*, pGAD-*ScGPR1*<sup>679-961</sup>, pGADT7-T, pGAD-*P53*<sup>72-390</sup>, and pGAD-*lam*<sup>66-230</sup> can not directly interact with pGBK-*PbCYR1*<sup>1-678</sup>.



### 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  $\alpha$  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  $\alpha$  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*<sup>679-961</sup>-RM-Lib with pGBK-*PbGPA1*, 2, and 3. Yeast-2-hybrid results indicated that *Pb* G protein  $\alpha$  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*<sup>679-961</sup>-RM-Lib.**

	pGBK- <i>PbGPA1</i>	pGBK- <i>PbGPA2</i>	pGBK- <i>PbGPA3</i>	pGBK- <i>ScGPA2</i>
pGAD- <i>ScGPR1</i> <sup>679-961</sup> -RM-Lib	0	0	0	50

A positive control was made by screening pGAD-*ScGPR1*<sup>679-961</sup>-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  $\alpha$  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- <i>PbCYR1</i> <sup>1-678</sup>	pGBK- <i>PbCYR1</i> <sup>600-1316</sup>	pGBK- <i>PbCYR1</i> <sup>1302-1876</sup>	pGBK- <i>PbCYR1</i> <sup>1648-2100</sup>
pGAD- <i>PbCYR1</i> <sup>1-678</sup>	–	–	–	–
pGAD- <i>PbCYR1</i> <sup>600-1316</sup>	Not tested	–	–	–
pGAD- <i>PbCYR1</i> <sup>1301-1876</sup>	Not tested	Not tested	–	–
pGAD- <i>PbCYR1</i> <sup>1347-2100</sup>	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*<sup>1-678</sup> 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*<sup>1648-2100</sup>, 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 $\gamma$ Subunit PbGpg1 Interacts with Truncated Adenylate Cyclase PbCyr1<sup>1-678</sup> and Truncated $\beta$ Subunit PbGpb1 but not Full-Length $\beta$ Subunit PbGpb1 and Full-Length $\alpha$ Subunits PbGpa1-3

A G protein  $\gamma$  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  $\gamma$  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  $\alpha$ -helical polypeptide that is supposed to form a stable dimer with the G protein  $\beta$  subunit but not to contact with the  $\alpha$  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<sup>1-678</sup> but not full-length PbGpb1 and full-length PbGpa1-3.**

Constructions for PbGpg1	Constructions for PbCyr1, PbGpb1 and pbGpa1-3 (see Table 3.11)				
	pGBK- <i>PbCYR1</i> <sup>1-678</sup>	pGBK- <i>PbGPB1</i>	pGBK- <i>PbGPA1-3</i>	pGAD- <i>PbGPB1</i>	pGAD- <i>PbGPA1-3</i>
pGAD- <i>PbGPG1</i>	+	-	-	Not applicable	Not applicable
pGBK- <i>PbGPG1</i>	Not applicable	Not applicable	Not applicable	-	-

The results indicated that PbGpg1 interacts with PbCyr1<sup>1-678</sup> 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- <i>PbGPB1</i> WD1	WD40 domains 2-7 were deleted.
pGAD- <i>PbGPB1</i> WD1-2	WD40 domains 3-7 were deleted.
pGAD- <i>PbGPB1</i> WD1-3	WD40 domains 4-7 were deleted.
pGAD- <i>PbGPB1</i> WD1-4	WD40 domains 5-7 were deleted.
pGAD- <i>PbGPB1</i> WD1-5	WD40 domains 6-7 were deleted.
pGAD- <i>PbGPB1</i> WD1-6	WD40 domain 7 was deleted.
pGAD- <i>PbGPB1</i> WD2-7	WD40 domain 1 and coiled-coil domain were deleted.
pGAD- <i>PbGPB1</i> 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- <i>PbGPB1</i> WD1	pGAD- <i>PbGPB1</i> WD1-2	pGAD- <i>PbGPB1</i> WD1-3	pGAD- <i>PbGPB1</i> WD1-4	pGAD- <i>PbGPB1</i> WD1-5	pGAD- <i>PbGPB1</i> WD1-6	pGAD- <i>PbGPB1</i> WD2-7	pGAD- <i>PbGPB1</i> WD6-7
pGBK- <i>PbGPG1</i>	-	+	+	+	+	-	-	-

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  $\gamma$  subunit *PbGpg1* interacts with the

truncated  $\beta$  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- <i>PbGPA1</i>	pGBK- <i>PbGPA2</i>	pGBK- <i>PbGPA3</i>	pGBK- <i>PbGPB1</i>
pGAD- <i>PbGPA1</i>	Not tested	Not tested	Not tested	-
pGAD- <i>PbGPA2</i>	Not tested	Not tested	Not tested	-
pGAD- <i>PbGPA3</i>	Not tested	Not tested	Not tested	-
pGAD- <i>PbGPB1</i>	-	-	-	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- <i>PbGPA1</i>	pGBK- <i>PbGPA2</i>	pGBK- <i>PbGPA3</i>	pGBK- <i>PbGPB1</i>
pGAD- <i>PbGPA1</i> -RM-Lib	Not tested	Not tested	Not tested	0
pGAD- <i>PbGPA2</i> -RM-Lib	Not tested	Not tested	Not tested	0
pGAD- <i>PbGPA3</i> -RM-Lib	Not tested	Not tested	Not tested	0
pGAD- <i>PbGPB1</i> -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- <i>PbGPB1</i> WD1	pGAD- <i>PbGPB1</i> WD1-2	pGAD- <i>PbGPB1</i> WD1-3	pGAD- <i>PbGPB1</i> WD1-4	pGAD- <i>PbGPB1</i> WD1-5	pGAD- <i>PbGPB1</i> WD1-6	pGAD- <i>PbGPB1</i> WD2-7	pGAD- <i>PbGPB1</i> WD6-7
pGBK- <i>PbGPA1</i>	-	+	+	+	-	-	-	-
pGBK- <i>PbGPA2</i>	-	-	-	-	-	-	-	-
pGBK- <i>PbGPA3</i>	-	-	-	-	-	-	-	-

The results indicate that truncated G protein  $\beta$  subunit *PbGpb1* interacts with full-length  $\alpha$  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  $G\alpha$  gene (Nakafuku et al, 1987 and 1988); fission yeast *gpa2* gene was cloned by using the  $G\alpha$  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, introns 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  $\alpha$  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. grisea*, 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 G $\gamma$  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 marneffeii*. However, the biological behaviors of these fungi are diverse including geographical distributions. *A. nidulans* is only a weak opportunistic fungal pathogen. *P. marneffeii* 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<sup>Δ58</sup> and PbCyr1<sup>Δ58-64</sup>. 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<sup>Δ58-64</sup> 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, protzoans, 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 Gas or Gai 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  $\alpha$  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  $\alpha$  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  $\alpha$  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 G $\alpha$  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* G $\alpha$  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<sup>16</sup>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 G $\alpha$  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* G $\alpha$  proteins isolated here retain these consensus sequences. They are MGCGMS for PbGpa1, MGCASS for PbGpa2 and MGGCCS for PbGpa3. Myristoylation of G $\alpha$  proteins is responsible for membrane localization and coupling to the membrane receptor but does not affect the interaction with G $\beta\gamma$  (Song et al, 1996). Therefore, the 3 G $\alpha$  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 *PbTpk1* 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 flocculence, 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 *TUPI* 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 *TUPI* 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  $\beta$  subunit PbGpb1 (13% identity), suggesting a similar function to the G $\beta$  subunit. Indeed the structure of the *S. cerevisiae* Tup1 contains a seven-bladed  $\beta$  propeller which can be superimposed over the similar structure of G $\beta$  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<sup>G132V</sup> can interact with full-length Ime2 but an inactive Gpa2<sup>G299A</sup> 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 G $\alpha$ proteins and a G $\beta$ Protein Interact with Adenylate Cyclase in *Paracoccidioides brasiliensis***

The cAMP signaling pathway of *Paracoccidioides brasiliensis* is very important as demonstrated by the fact that all 3 G protein  $\alpha$  subunits, the G protein  $\beta$  subunit and  $\beta\gamma$  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 G $\alpha$  proteins can directly interact with adenylate cyclase in an organism. Our study is also the first to demonstrate that G $\beta$  and the G $\beta\gamma$  dimer can directly interact

with adenylate cyclase 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  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits, exist as inactive heterotrimers with GDP bound to the  $\alpha$  subunit and are coupled to a cell surface receptor (Hoffman, 2005a). Receptor stimulation leads to a conformational change in G $\alpha$  and exchange of GDP for GTP on the G protein  $\alpha$  subunit, activating G $\alpha$ . The activated G $\alpha$  then dissociates from the  $\beta\gamma$  subunits, which do not undergo a conformational change. Both  $\alpha$  and  $\beta\gamma$  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  $G\alpha$ , which then reassociates with free  $\beta\gamma$  to return to the inactive  $G\alpha\beta\gamma$  state. The duration of G protein signalling depends on the intrinsic GTPase rate of the  $G\alpha$  subunit but is also modulated by extrinsic factors such as the  $G\beta\gamma$  dimer and RGS proteins (regulators of G protein signalling; Hoffman, 2005a).

In mammalian cells, an excess of the  $G\beta\gamma$  dimer promotes reassociation with  $G\alpha$ , inhibiting  $G\alpha$  stimulated adenylate cyclase activity. In addition,  $G\beta\gamma$  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  $G\beta\gamma$  dimer that activates the pathway. The  $G\alpha$  subunit turns off the signal by association with the  $G\beta\gamma$  dimer. The G protein  $\beta$  subunit Ste4 is probably the limiting component of the active  $G\beta\gamma$  dimer because the activated pheromone response generated by overexpression of Ste4 ( $\beta$ ) was abolished by deletion of Ste18 ( $\gamma$ ) but overexpression of Ste18 alone does not induce the pheromone response (Cole et al, 1990). In addition, overexpression of Gpa1( $\alpha$ ) neutralizes the biological effect of overexpression of the  $\beta$  subunit Ste4 (Cole et al, 1990). Deletion of *GPA1* leads to the same phenotype as the overexpression of the  $\beta$  subunit Ste4, i.e. haploid-specific cell cycle arrest, indicating that  $G\beta\gamma$  is constitutively active without damping by the  $G\alpha$  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  $\alpha$ ,  $\beta$ , and  $\gamma$  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 ( $\beta$ ) is probably lower than that of Ste18 ( $\gamma$ ). Overexpression of Ste4 further potentiates the pheromone response, while overexpression of Gpa1 sequesters the  $G\beta\gamma$  dimer into an inactive  $G\alpha\beta\gamma$  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 (Metodieiev et al, 2002; Blackwell et al, 2003). Recently, it has also been found that the GTPase-deficient *GPA1*<sup>Q323L</sup> 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  $G\beta\gamma$ . Therefore, either  $G\alpha$  or  $G\beta\gamma$  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  $G\beta$  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( $\alpha$ ), PbGpb1( $\beta$ ) and PbGpg1( $\gamma$ ). The latter two subunits form a tight G $\beta\gamma$  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 G $\beta$  (or G $\beta\gamma$  dimer) modulate the same effector, i.e. adenylyate cyclase. The working mechanism is probably analogous to that in the pheromone pathway of *S. cerevisiae*. For example, PbGpb1-PbGpg1 could activate the adenylyate cyclase while PbGpa1 could negatively regulate adenylyate cyclase, and meanwhile sequester the G $\beta\gamma$  dimer and inactivate it. Alternatively, PbGpa1 may play the active role while the *Pb* G $\beta\gamma$  dimer may play the negative role by interacting with both adenylyate 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, adenylyate 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 adenylyate cyclase. It is also possible that both Gpa1 and the PbGpb1-PbGpg1 dimer activate adenylyate cyclase, while one sequesters another by direct interaction.

The ratio of PbGpa1 and the G $\beta\gamma$  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 process, 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  $\alpha$  subunit PbGpa1 and the  $\beta$  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 phosphatidylcholine transferases as chaperones for the G $\beta\gamma$  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 G $\alpha\beta\gamma$  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 cyclase 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 adenylate cyclase *Cyr1*<sup>K1876M</sup> 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( $\alpha$ ), Ste4( $\beta$ ) and Ste18( $\gamma$ ). The G $\alpha$  can directly interact with G $\beta\gamma$  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 G $\alpha$  and G $\beta\gamma$ , 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 G $\alpha$  changes its conformation when dissociated from the G $\beta\gamma$  but that G $\beta$  does not change its conformation following dissociation (Lambright et al, 1996). The G $\beta$  folds into a symmetric propeller structure containing seven blades of  $\beta$  sheets. For each blade, there are 4 antiparallel  $\beta$  sheets radiating outwards from a central axis. The first 3  $\beta$  sheets of an individual blade come from the C-terminus of a WD40 sequence but the fourth  $\beta$  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<sup>th</sup> blade. The N-terminal region of G $\beta$  is involved in the coiled-coil interaction with the N-terminus of G $\gamma$ . The G $\beta\gamma$  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  $\beta$  and  $\gamma$  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 phosphatidylcholine transferase, for assistance in G $\beta\gamma$  dimer assembly (Lucov et al, 2005; Chen et al, unpublished data). It is interesting that the G $\gamma$  PbGpg1 and the G $\alpha$  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 from 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 G $\beta\gamma$  dimer, the down-regulation of Hsp30 may make Hsp30 unavailable for G $\alpha$  and G $\beta$ .

Without the assistance of Hsp30, G $\alpha$  and G $\beta$  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 G $\beta\gamma$  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 interact 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 cyclase 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 cyclase, 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 *ras1Δ* or *ras2Δ* 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<sup>ts</sup>* (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 length 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  $G\alpha$  dissociates from  $G\beta\gamma$  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  $G\alpha$  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 downstream effectors of  $G\alpha$  and  $G\beta\gamma$  are different. Our data indicates that in *Pb*, both the  $G\alpha$  and  $G\beta\gamma$  directly work on the N-terminal end of adenylate cyclase. It would be interesting to crystallize the  $G\alpha(\text{GDP})$ -adenylate cyclase dimer,  $G\alpha(\text{GppNHp})$ -adenylate cyclase dimer and  $G\beta$ -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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