INVESTIGATION OF CLONING STRATEGIES for *A. thaliana* G PROTEIN α-SUBUNIT GENE in *Pichia pastoris*

by
BURCU KAPLAN

Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Master of Science

Sabancı University
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APPROVED BY:

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GPA1 was amplified by PCR and cloned using a *Pichia pastoris* expression system. Two different plasmids pPICZC+*GPA1* and pPICZαB+*GPA1'* were constructed. pPICZC+*GPA1* was designed for intracellular expression whereas pPICZαB+*GPA1'* contained a signal peptide facilitating secretion of the recombinant protein into the extracellular medium. The possibility of using different yeast strains that may improve expression was explored. Recombinant synthesis of GPA1 was achieved with the pPICZC+*GPA1* construct using the strain GS115, which shows Mut^+ phenotype. Expression was followed by monitoring growth of yeast as well as western blots of cellular extracts at different time points during induction.

This study describes the first report of expression of *A. thaliana GPA1* gene in a eukaryotic system and constitutes a critical step forward in studies of G-proteins in plants. It follows to reason that the availability of purified recombinant GPA1 will enable biochemical characterization, comparison with its mammalian counterparts and facilitate structural studies.

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Bu tezde A. thaliana heterotrimerik G-proteini α alt birimi geninin klonlanması ve ifadesi için yapılan çalışmalar sunulmuştur. Bu amaç doğrultusunda bol miktarda ve yüksek saflıkta rekombinant protein üretimi için uygun bir ökaryotik ifade hücresi seçilmiştir.

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1 INTRODUCTION

Heterotrimeric G proteins are mediators that transmit the external signals via receptor molecules to effector molecules and play a crucial role in signal transduction in mammalian and plant systems. Biochemical and molecular evidence point to involvement of G proteins in various plant processes such as phytochorme, auxin, absisic acid or blue light signaling and in plant defense mechanisms (Ma, 2001). The mammalian heterotrimeric G proteins are composed of three subunits, α, β and γ. The α subunit by binding /dissociation from βγ dimer, transmits signals from receptor to effector molecules. In plants studies revealed the presence of the three subunits in the *A. thaliana* genome. The G protein α subunit, GPA1 was first to be isolated (Ma *et al.,* 1990) followed by the β- subunit AGB1 (Weiss *et al.,* 1994) and the two γ-subunits; AGG1, AGG2 (Mason and Botella, 2000 and 2001).

Although mammalian heterotrimeric G proteins are well characterized, studies on plant systems are limited. Recently the presence of a G protein coupled receptor, GCR1, in *A. thaliana* was suggested based on the evidence of interaction of the G protein α subunit with the receptor (Pandey and Assmann, 2004). The presence of a G protein coupled receptor (GPCR) raises the possibility that the mechanism of action of the heterotrimer in plants is similar to that observed in mammalian systems. Interaction of the subunits β and γ have been reported based on yeast two hybrid studies (Mason and Botella, 2000 and 2001), but there is no direct experimental evidence for an interaction between the α subunit and the βγ dimer, and hence a mechanism involving the activation of the heterotrimer through dissociation of α subunit from the βγ dimer followed by the exchange of GDP with GTP.

Our aim is to produce significant quantities of *A. thaliana* G protein α subunit (Gα) for biochemical characterization and structural studies. Stuctural characterization

of Gα will reveal the level of similarity with the mammalian counterpart and may allow prediction of the nature of interactions with the βγ complex in plants. For this purpose the gene encoding Gα*, GPA*1 is cloned and expressed in yeast *Pichia pastoris*. Expression conditions were investigated and the preliminary characterization of the recombinant protein has been carried out. The possibility of expressing β and γ subunits using the same expression system is investigated. The recombinant proteins will be isolated for X-ray solution scattering and crystallography. A comparison of structural techniques shows that, small angle scattering from proteins in solution is helpful for determining protein-protein interactions and domain movements during interactions. X-ray crystallography would provide high resolution static information on the structure in the crystallized form of the protein, whereas NMR is more easily applicable to small molecules. In addition solution X-ray scattering allows monitoring the dynamics of conformational changes due to interactions of proteins in solution. Structural data from plant heterotrimeric G proteins is necessary for meaningful comparison with mammalian homologs and functional attributions based on this comparison.

There is limited literature on recombinant expression of plant Gα subunits;, cloning of *A. thaliana GPA1* has been reported by Wise *et al* (1997) and that of rice RGA1 by Iwasaki *et al*. (1997) and Seo *et al*. (1995). The initial study on cloning of *GPA1* in *Escherichia coli* had reported a yield of 1–2 mg of recombinant Gα from 1 litre of liquid culture (Wise *et al*., 1997). In this study pUBS520 plasmid encoding for arginine tRNA, which are of low abundance in E. *coli*, was used in order to prevent premature termination of the translated protein. A more recent study focused on cloning of *GPA1* in *E. coli*, using different expression vectors; here possible effects of using a prokaryotic host for expression were reported. Besides the lack of eukaryotic posttranslational modifications in the host cell, toxic effect of the plant protein leading to either its degradation/ truncation was considered (Bakkal, 2003). In the light of the above mentioned observations, in the present study a eukaryotic expression system was chosen to clone and express the target plant protein.

Pichia pastoris, a methylotrophic yeast, has the advantages of an eukaryotic experimental organism such as ease of genetic manipulation, ability to perform post-translational modifications of eukaryotic proteins and allows large-scale production in

fermentation systems (Hollenberg and Gellissen,1997). Furthermore the strong promoter of *AOX1* gene helps to avoid toxic effects of heterologous protein expression until expression of the product is induced by methanol. In this study two different expression vectors and two *P. pastoris* strains were used. The expression vectors are yeast integrative plasmids, which recombinate into yeast genome via shared sequences. Important features of the plasmids include the promoter of *AOX1* gene, as the homologous sequence with the yeast genome, the transcriptional termination sequence of *AOX1* gene for efficient processing and polyadenylation of mRNAs, a multiple cloning site (MCS) for the insertion of the foreign gene between the two AOX regions, c-*myc* epitope and His-tag downstream of MCS for analysis and purification of the recombinant protein, a PUC ori for maintenance and replication in bacterial hosts and finally the zeocin resistance gene which functions as selectable marker in both bacteria and yeast. One of the expression vectors contain the secretion signal sequence from the *Saccharomyces cerevisiae* α factor prepro peptide which leads to secretion of the recombinant protein into external medium.

The cloning strategies performed and details of preliminary studies of expression of recombinant Gα are presented in this thesis. This is the first study of cloning and expression of the *A. thaliana* Gα in an eukaryotic expression system. Next steps involve purification, GTP-binding, GTPase activity verification and structural characterization of recombinant A. thaliana Gα. Expression and characterization of β and γ subunits of *A. thaliana* G protein will contribute to the understanding of heterotrimeric G protein signaling in plants.

2 OVERVIEW

2.1 Heterotrimeric G proteins and G protein α Subunits in Plants

2.1.1 The Heterotrimer

In all eukaryotes, including primitive unicellular organisms, GTP binding proteins play important roles in the specificity and modes of cellular responses to extra cellular signals. In the mammalian systems the heterotrimeric protein complex is made up of three subunits labeled as α-, β- and γ-. Along with the heterotrimer the receptor (GPCR) and effector molecules function in signal transduction in the upstream and downstream processes, respectively. G proteins are inactive in the GDP-bound, heterotrimeric state and are activated by receptor-catalyzed guanine nucleotide exchange resulting in GTP binding to the α subunit (Gα). GTP binding leads to dissociation of GαGTP from Gβγ subunits and activation of downstream processes.

The mammalian α subunit has two domains, one with an α helical secondary structure with unknown function and the other a ras domain. Ras domain contains the GTP/GDP binding site, the GTP hydrolase activity, the covalently attached lipid anchoring the subunit to the bilayer and the backbone loops which act as switches depending on the bound nucleotide. Upon activation of GPCR, with the binding of the ligand to the extracellular binding site, membrane bound Gα inside the cell interacts with GPCR. This interaction, occurring between the cytoplasmic loop of the receptor and the amino- and carboxy terminal domains of Gα catalyses the nucleotide exchange.

The nucleotide exchange, GDP to GTP, releases Gα from Gβ and this binding site upon a conformational change is filled with effector molecules, such as; adenylyl cyclases and cGMP phosphodiesterase. Gβ, released from Gα, remains strictly bound to the γ subunit which anchors the heterotrimer / dimer to the lipid bilayer via lipid modification at its carboxy terminus. The intrinsic GTPase activity of Gα eventually results in GTP hydrolysis and in the reformation of the heterotrimer. The free dimer interacts with several downstream molecules depending on the activating receptor; such as, phospholipase Cβ, adenylyl cyclases, Na^+ and K^+ ion channels and a variety of serine/threonine kinases (Clapham and Neer,1993). Both Gα and Gβγ are regulated by other proteins; 'regulators of G-protein signaling' (RGS). RGS, which are GTPase activating proteins in the case of Gα (GAPs), bind to Gα and accelerate the rate of GTP hydrolysis to GDP, shortening the lifetime of Gα's active, GTP-bound state. GAPs lead to reduced signal strength and/or accelerated termination of the signal after ligand removal from the GPCR. Gβγ subunits are regulated by phosducin, a protein that tightly binds to the dimer and prevents interaction with Gα and/or downstream effectors (Willardson *et al*., 1996).

The signal transduction pathways involving heterotrimeric GTP binding proteins are summarized in figure 2.1 (Bohm *et al*., 1997).

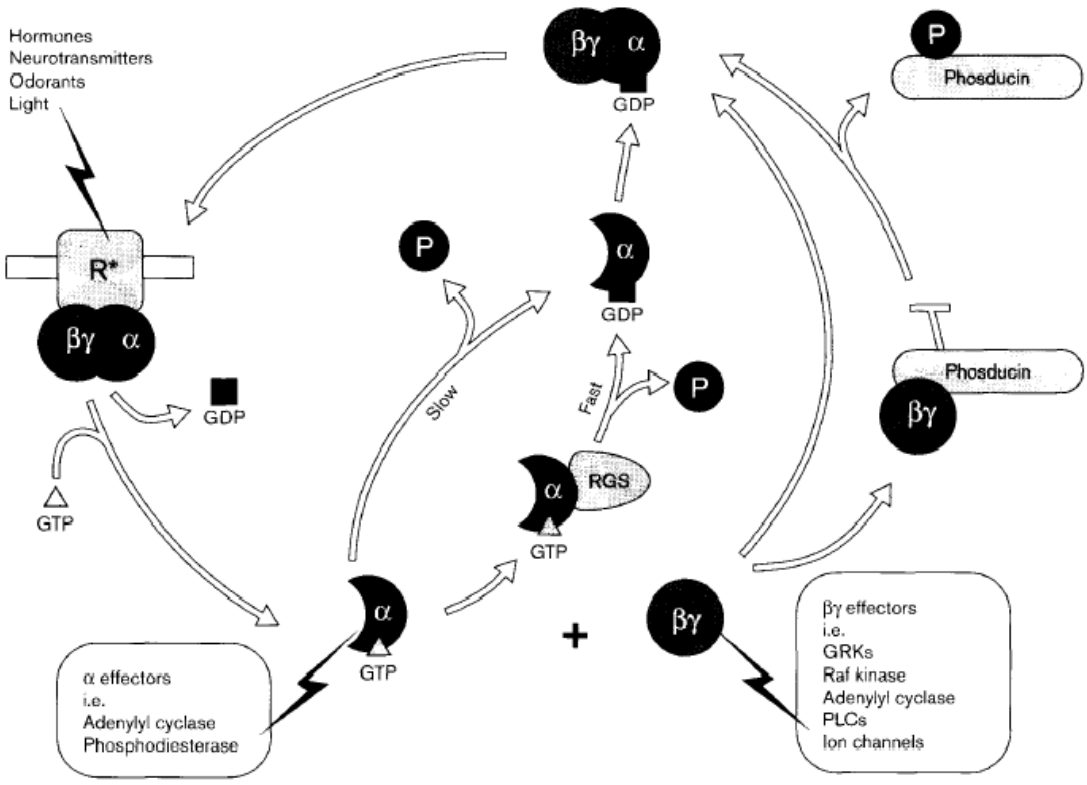


Figure 2.1Schematic diagram of G-protein coupled signal transduction pathways

In mammalian systems there are 23 Gα, 6 Gβ, 12 Gγ subunits together with a large number of isomer specific receptor and effector molecules. The four subfamilies of Gα are; G_s, which activates adenylyl cyclase; G_i, which inhibits adenylyl cyclase; G_q, which activates phospholipase C; and $G_{12/13}$, of unknown function (Hamm, 1998). $G\alpha_{12}$ and $G\alpha_{13}$ appear to participate in cell transformation and embryonic development, but the signaling pathways that are regulated by these proteins have not been identified.

2.1.2 The heterotrimer in *Arabidopsis thaliana*

Like their animal counterparts, the heterotrimeric G proteins of *Arabidopsis* consist of three subunits, the so-called Gα, Gβ and Gγ subunits. In contrast to animals, *Arabidopsis* has only one canonical Gα gene, *GPA1* (Ma *et al.*, 1990), one Gβ gene, *AGB1* (Ma, 1994; Weiss *et al.*, 1994), and two Gγ genes, *AGG1* and *AGG2* (Mason and Botella, 2000, 2001). Molecular modeling suggests an interaction between GPA1 and

AGB1 (Ullah *et al*., 2003) while a strong interaction between AGB1 and AGG1 or AGG2 was detected by yeast-two hybrid and in vitro binding assays (Mason and Botella, 2000, 2001).

2.1.2.1 The α subunit

The *A. thaliana* Gα was isolated by a PCR approach using degenerate oligonucleotides derived from two highly conserved regions of mammalian and yeast G protein α subunits, followed by isolation of genomic cosmid clones with the PCR products as template. The resulting gene was 1149 bp with a predicted protein of 383 amino acids, corresponding to a molecular weight (MW) of 44,482 Da (Ma H, 2001).

Gα is detected in all organs, and cell types being most abundant in vegetative tissues including leaves and roots, less in floral stems and least in floral buds and floral meristems (Ma *et al*., 1994; Ma H, 2001). Results of localization studies of Gα are consistent with the classical heterotrimer model where the protein immunolocalizes at the plasma membrane and endoplasmic reticulum (ER) membrane (Weiss *et al*., 1997).

Studies with recombinant Gα subunits are limited, though they give information about the function. The $GTP_{\gamma}S$ binding constant for purified recombinant *A. thaliana* Gα, expressed in *E. coli*, was reported to be 0,34 nM, assuming one binding site (Wise *et al*.,1997). The gene encoding for rice Gα was cloned and expressed in *E. coli* and purified recombinant protein was shown to bind to $GTP_{\gamma}S$ with an apparent binding constant of 0,36 nM, without Mg^{+} requirement (Iwasaki *et al*., 1997). The presence of Mg^{+} is strictly required for the binding of mammalian G protein α subunits to $GTP_{\gamma}S$, with the exception of $G\alpha_z$ (Casey *et al*., 1990). Recombinant rice Gα resembles $G\alpha_z$ by binding to $GTP_{\gamma}S$ even in the absence of Mg^{+} and binding is slightly enhanced in the presence of Mg^{+}. The GTPase activity analyses yielded a k_{cat} value (0,44 min^{-1}) smaller than those of mammalian counterparts except $G\alpha_z$ (0.05 min^{-1}) (Iwasaki *et al*., 1997).

Analyzing the similarity between mammalian counterparts, Gα is 36% identical to G_i subfamily considering the critical domains involved in activation of mammalian heterotrimeric protein. The similarity results from the unusual myristoylation motif and absence of the carboxy-terminal cysteine of the $G\alpha_z$, which is known to play a role in **cell proliferation** and death via its control of **potassium channeling** (Jones, 2002).

Arabidopsis mutants, generated by T-DNA insertion into the *GPA1* (gpa1), have reduced **cell division** during hypocotyl and leaf formation (Ullah *et al*., 2001), furthermore it has been suggested that *GPA1* is involved in promoting active cell division (Ma H, 1994). The high levels of *GPA1* expression reported in meristematic tissue (Weiss *et al*., 1997) is consistent with a role for *GPA1* in cell division. Tobacco cells over expressing *GPA1* progress more rapidly through cell cycle, while control cells required auxin to reach the level of cell division of over expressing cells. It is well known that the plant hormone auxin regulates cell division, but auxin-induced cell division still occurs, although sensitivity to the hormone is altered, in mutants lacking either Gα or Gβ, thus indicating that auxin can not be directly coupled by a G protein (Ullah *et al*., 2001). The control of cell cycle regulation is coupled somehow to heterotrimeric G proteins but the details remain unknown.

In plants, guard cell ion-channel regulation controls stomatal apertures. During stomatal opening, K^+ uptake is mediated by inwardly rectifying **K^+ channels**. During inhibition of stomatal opening by the plant hormone abscisic acid (ABA), these channels are inhibited, by the activation of phospholipases C and D in the guard cells. It is known that certain phospholipases C and D are regulated by heterotrimeric G proteins in mammalian systems. Interestingly a similar regulation is observed for the *Arabidopsis* guard cells, where *GPA1* was shown to be expressed (Wang *et al*., 2001). ABA inhibition of light induced stomatal opening or inward K^+ channels are lacking in gpa1 mutants. Furthermore ABA does not activate pH-independent anion channels in gpa1 mutants. Besides, the pH-dependent pathway of ABA action is unaffected in gpa1 mutants, suggesting the presence of different ABA pathways either including *GPA1* or not. This multiplicity in signaling is also observed when different cell type mutants are compared. Unlike guard cells gpa1 seeds posses wildtype sensitivity to ABA. But these mutants are less sensitive to gibberelic acid (GA) and completely insensitive to

brassinosteroid (BR), while overexpression of *GPA*1 results in hypersensitivization to GA; still requiring GA for seed germination (Ullah *et al*., 2002).

Although there are many signaling pathways that have been shown to involve Gα, the involvement in cell proliferation and cation channels highlight the similarities with mammalian Gα, supported with the (sequence) conservation of functional domains. There are, however, also several differences including lack of isomeric diversity of heterotrimeric subunits and lack of receptors in plants. Only one possible GPCR, designated as GCR1, has been identified from *A. thaliana*. Recently GCR1 has been shown to interact with Gα by *in vitro* pull-down assays, by yeast split-ubiqiutin assays and by co immunoprecipitation from plant tissue, but a ligand for GCR1 has not been defined yet (Pandey and Assmann,2004). The C-terminal domain of all known plant Gα is nearly 100% conserved unlike in the case of mammalian Gα's, where this region is poorly conserved due to diversity in Gα/receptor interactions. The high conservation at the receptor binding site may indicate that there is a single / only a few receptor(s) with which plant Gα can interact.

The high level of sequence identity for Gα among plant species is also observed for Gβ subunit. As a matter of fact identification and characterization of plant heterotrimeric G proteins are mainly based on sequence homology with their mammalian counterparts. Mutant plant studies verified the assigned functional roles and possible pathways involved (Fujisawa *et al*., 2001). All the characterized plant Gα proteins are given in table 2.1, together with samples of characterized plant β- and γ-subunits. The sequence homology of *A. thaliana* Gα with all structurally characterized G protein α subunits was analyzed in order to model the structure of Gα. and the members of transducin family , especially rat (*rattus norvegicus*), were shown to yield highest scores of PSIBLAST search (Şahin, 2002). The transducin family is classified within G_i subfamily of mammalian Gα subunits. But the function related characteristics of plant Gα subunits are more similar to G_z, another member of G_i subfamily. Amino acid sequence conservations between *A. thaliana* Gα and rat transducin lie within the Gβγ binding and GTP-hydrolysis domains of rat transducin. These key residues are also nearly fully conserved among known 14 plant Gα subunits. Based on this homology 3D

structural models of *A. thaliana* Gα was generated and optimized to yield the final model, shown in figure 2.2 A (Şahin, 2002).

The model, with the conserved residues and motifs at the functional domains, supports biochemical data that *A. thaliana* Gα possess GTPase activity and a binding site for GDP/GTP, since mixed α-helical/β-strand Ras-like domain with GTPase function and GTP binding pocket are conserved. Yet the conformation of switch regions upon nucleotide exchange should be investigated using a dynamic approach such as solution X-ray scattering and/or NMR. In mammalian Gα, GTP binding brings switch regions to close contact with each other, whereas as in the GDP bound state the switches are more flexible, allowing the interaction of the subunit with Gβγ dimer. The N-terminal helix region ,purple in figure 2.2.B, is buried in the core of the protein when GTP is bound, when switch II is free of the nucleotide's γ-phosphate the N-terminal helix region interacts with Gβγ and it is drawn away from the bulk of Gα (Bohm *et al*., 1997). The predicted model will be informative for analyzing X-ray data of a recombinant plant Gα, but conclusions on the model should be made with care since the template used was not the functional homolog $G\alpha_z$.

A

B

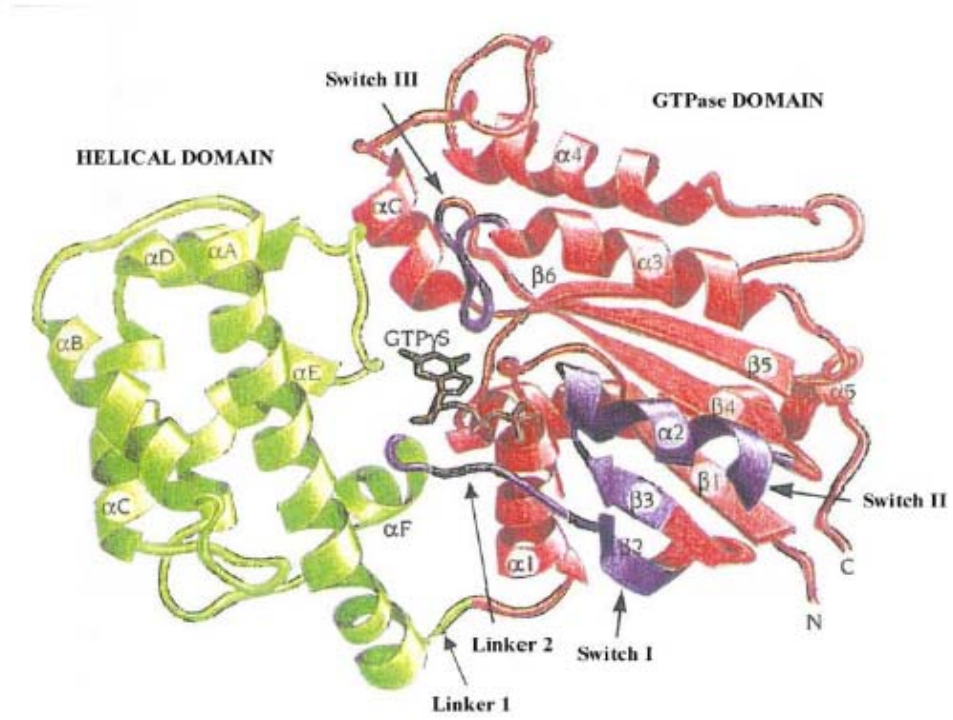


Figure 2.2 A: Model of G protein α subunit (Şahin, 2002) B: Overall structure of GDP bound G protein α subunit (Rens-Domiano S *et al., 1995).*

Gene	Species	Classification	References
GPA1	Arabidopsis	G_{α}	Ma *et al*., 1990
LjGPA1	Lotus	G_{α}	Poulsen *et al*., 1994
LGPα1	Lupin	G_{α}	Kusnetsov and Oelmueller, 1996 b
NPGPA1	Nicotiana plumbaginifolia	G_{α}	Kaymadow *et al*.,2000
PGA1,PGA2	Pea	G_{α}	Marsh *et al*., 1999
RGA1	Rice	G_{α}	Ishikawa *et al*., 1995; Seo *et al*., 1995
SGA1	Soybean	G_{α}	Kim *et al*.,1995
SGA2	Soybean	G_{α}	Gotor *et al*., 1996
SOGA1	Spinach	G_{α}	Perroud *et al*., 2000
NtGPa1	Tobacco	G_{α}	Saalbach *et al*., 1999
NtGA2	Tobacco	G_{α}	Ando *et al*.,2000
TGA1	Tomato	G_{α}	Ma *et al*., 1991
AfGα1	Wild oat	G_{α}	Jones *et al*., 1998
AGB1	Arabidopsis	G_{β}	Weiss *et al*., 1994
RGB1	Rice	G_{β}	Ishikawa *et al*., 1996
ZGB1	Maize	G_{β}	Weiss *et al*., 1994
AGG1,	Arabidopsis	G_{γ}	Mason, M.G., and Botella, J.R. (2000)
AGG2	Arabidopsis	G_{γ}	Mason, M.G., and Botella, J.R. (2001)

Table 2.1 Subunits of heterotrimeric G proteins isolated from different plants. (Assmann, 2002 and references therein)

2.1.2.2 The Gβγ complex

*AGB1*and *ZGB1* and are the heterotrimeric G-protein β-subunit genes which were isolated from *A. thaliana* and maize, respectively. They are approximately 41% identical with animal G protein β-subunits and contain seven copies of WD40 motif, which is the common property of β-subunits. There is 76% similarity between the two genes suggesting a similar function for the translated proteins. According to the expression patterns tested by Northern hybridization, *AGB1* was detected in the root, leaf and the flower (Weiss *et al.,* 1994).

The completion of the heterotrimer was achieved with the isolation of the two plant γ-subunits, *AGG1* and *AGG2* from *A. thaliana*. These two small proteins posses the conserved characteristics of γ-subunits like small size, C-terminal CAAX box and N-terminal α-helix region capable of forming a coiled-coil interaction with β-subunit. These genes code for a 98 amino acid peptide with a molecular weight of 10.8 kDa. As in the case of *AGB1*, *AGG1* and *AGG2* are mainly expressed in roots, leaves and flowers. Results of experiments using a yeast two-hybrid system, strong interaction of *AGB1* with *AGG1* and *AGG2,* has been defined. This indicates the importance of the coiled-coil domain of AGB1 for interaction with AGG1 (Mason and Botella, 2000; Mason and Botella, 2001). The *AGG1* gene was reported to be expressed in *E. coli* BL21(DE3) cells (Seckin, 2003).

2.1.3 Structure- function relations of heterotrimeric G proteins

The structure of proteins is an important reference for predicting function. In the case of heterotrimeric G proteins, it is well known that key structural domains regulate the function of the complex. All three subunits have characteristic functional regions conserved among structurally characterized mammalian proteins. There are experimentally–determined structures for two different mammalian G protein heterotrimers, a 2.0 Å structure of the heterotrimer Gt-α (bovine) /Gi-α (rat) chimera, Gi-β1 (human), Gt-γ1 (bovine) (Lambright *et al*., 1996), PDB accession code 1GOT and a 2.3 Å structure of the Gi-α1 (rat), Gi-β1 (human), Gi-γ2 (C68S) (bovine) (Wall *et al*., 1995), PDB accession code 1GP2.

The *A. thaliana* heterotrimer structure was modeled by Ulah *et al*. (2003) using the high resolution structure 1GOT as template for homology modeling. Each subunit was modeled independently and models were superimposed onto the heterotrimer structure. The model and template are shown in figure 2.3. The model is consistent with the Gα model of Şahin (2002), regarding the GTPase domain and the N –terminal helix, which is in contact with the Gβ subunit. The conformational change upon loss of γ-phosphate of GTP can be clearly observed comparing the free Gα (figure 2.2.B) and heterotrimeric form of Gα (blue in figure 2.3). A similar structure at the Gα /Gβ interface and the Gβγ dimer structure of the model and the crystal structure suggest that plant heterotrimeric G protein exists and activation by nucleotide exchange may follow the mammalian pattern by means of subunit binding/dissociation.

The conserved amino acid sequences and key functional domains based on structural models, the biochemical evidence for similar pathways involved and the activity assays verifying GTP binding strongly suggest that the plant heterotrimer is the structural and functional homolog of the key complex involved in mammalian signal transduction.

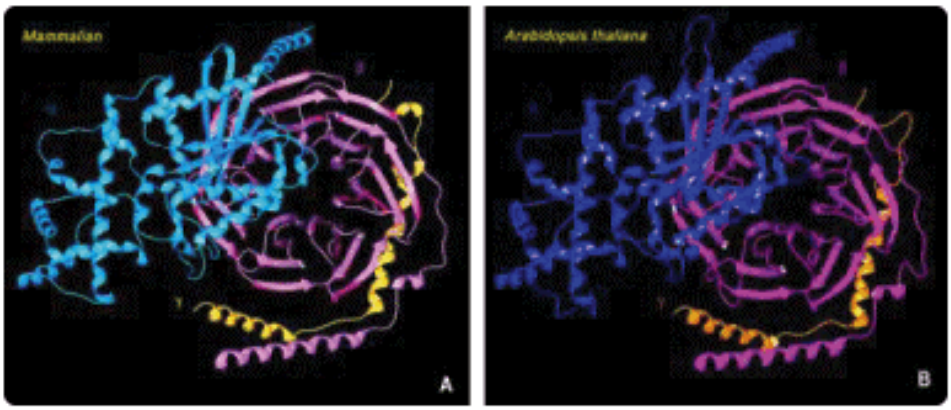


Figure 2.3 A comparison of the structure of the composite mammalian heterotrimeric G protein complex, PDB accession code 1GOT (A) and the modeled Arabidopsis complex (Ullah *et al*., 2003)(B).The α, β and γ subunits colored blue, purple and gold, respectively.

2.2 The Expression System: *Pichia. pastoris*

Yeasts are unicellular eukaryotic organisms, *S. cerevisiae* being the most commonly used system in biotechnological applications, including recombinant protein expression. Yeasts have the ability to perform eukaryotic-specific post-translational modifications such as proteolytic processing, folding, disulfide bridge formation, and glycosylation (Eckart and Bussineau, 1996). Bacterial expression systems lack these abilities and often produce misfolded, insoluble or inactive protein formation in inclusion bodies. Mammalian and baculovirus-infected cell lines, on the other hand, are not economic, difficult to handle and impractical for large-scale expression.

Pichia pastoris is a methylotrophic yeast which is being increasingly used as an alternative to S. *cerevisiae* in biotechnological applications during the past 20 years. *P. pastoris* has the properties of an eukaryotic experimental organism such as ease of genetic manipulation, its ability to perform post-translational modifications of eukaryotic proteins and allowing large-scale production in fermentation systems (Hollenberg and Gellissen,1997). Furthermore problems encountered in *S. cerevisiae* such as, mitotic instability of recombinant strains, a great extent of undesirable overglycosylation and difficulties in adapting expression to fermentation have been overcome with the introduction of *P. pastoris* (Gellissen and Hollenberg ,1997).

Pichia , unlike S. *cerevisia*e , with its preference to respiratory growth is a poor fermenter yielding high biomass in controlled environment of a fermenter; which is roughly proportional to the amount of secreted protein. *P. pastoris* can be grown to densities of 100 g/litre (dry weight) in continuous fermenter cultures, which are hard to reach with S. *cerevisiae* (Cregg *et al*., 1993). The highest yield reported in *P. pastoris* for an intracellularly expressed protein is 12 g/litre for tetanus toxin fragment C (Clare *et al*., 1991) whereas the highest yield reported for secreted proteins is 2.5 g/litre for bacterial α-amylase (Paifer *et al*., 1994).

Methylotrophic yeasts, of the genera *Candida, Hansenula, Pichia* and *Torulopsis,* can utilize methanol as the sole source of carbon and energy. Methanol induces a specific methanol utilization pathway leading to expression of key enzymes under the control of tightly regulated promoters. One of these key enzymes, alcohol oxidase (AOX), catalyses the oxidation of methanol to formaldehyde and hydrogen peroxide. The reaction takes place in peroxisomes, where hydrogen peroxide is degraded into oxygen and water by the activity of the enzyme catalase (Cereghino and Cregg, 2000).

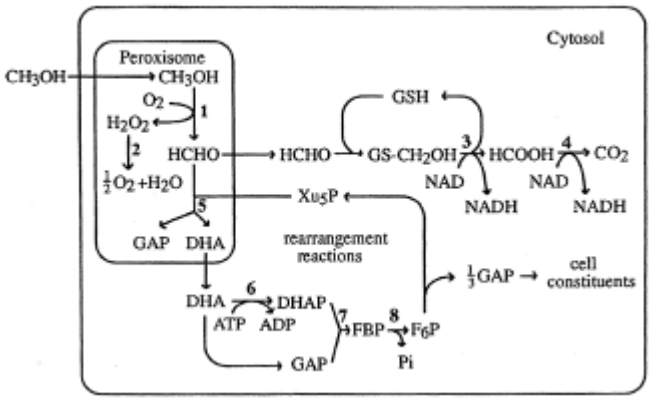


Figure 2.4 The methanol pathway in *P. pastoris*.
1:alcohol oxidase, 2: catalase, 3: formaldeyde dehydrogenase, 4: formate dehydrogenase, 5:dihydroxyacetone synthase, 6:dihydroxyacetone kinase, 7: fructose 1,6-biphosphate aldolase, 8: fructose 1,6-biphosphatase (Cereghino and Cregg, 2000).

AOX is encoded by two genes in *P. pastoris* namely, *AOX1* and *AOX2*, former being responsible for the majority of alcohol oxidase activity in the cell (Cregg *et al*., 1989). The *AOX*1 gene expression is controlled at the level of transcription and the presence of methanol is essential to induce high levels of transcipt. 5% of RNA isolated from methanol grown cells is from the *AOX*1 gene, whereas *AOX*1 message is undetectable in cells grown on any other carbon source (Tschopp *et al*., 1987). In fermenter cultures with methanol fed at growth limiting rates *AOX1* transcription levels can be as high as 30% of total soluble protein. Like the *S. cerevisiae GAL1* gene; *AOX1* gene is under the control of two mechanisms; repression/derepression and induction, but repressing carbon source, any source other than methanol, does not result in transcription of *AOX1* (Higgins and Cregg, 1998). The strong promoter of *AOX*1 avoids any toxic effects of heterologous protein expression until expression of the product is

induced by methanol. The phenotype of *P. pastoris* strains which can utilize methanol as a sole carbon source is designated as Mut^+.

*AOX*2 gene is weaker than the *AOX*1 gene and thus deletions in the *AOX*1 gene results in slower growth on methanol. *P. pastoris* strains KM71 and KM71H have a partial insertion in *AOX1* gene and thus rely only on *AOX*2 for methanol utilization. This strain grows slower than wild type strains on methanol showing Mut^s phenotype (methanol utilization slow phenotype). The deletion of both genes results in a strain that is unable to grow on methanol as the only carbon source and this phenotype is designated as Mut^- (methanol utilization minus phenotype). Deletion of AOX genes does not affect the strains ability to induce expression at high levels from the *AOX*1 promoter (Chiruvolu *et al*., 1997).

Some engineered strains have a mutation in the histidinal dehydrogenase gene (*HIS4*) and can not grow in minimal media unless supplemented with histidine. There are also protease deficient strains, of which different protease genes have been eliminated. Table 2.2 summarizes the genotypes and phenotypes of some of the available strains.

strain	Genotype	phenotype
X-33	wild type	Mut^+ His^+
GS115	his4	Mut^+ His^-
KM71	arg4 his4 aox1::ARG4	Mut^s His^- Arg^+
KM71H	arg4 aox1::ARG4	Mut^s His^+ Arg^+
GS115/Albumin	HIS4	Mut^s His^+
GS115/pPICZC/*lac*Z	his4	Mut^+ His^-
MC100-3	aox1::ARG4 aox2::Phis4 his4 arg4	Mut^- His^-
SMD1168	pep4Δ his4	Mut^+ His^- Protease deficient
SMD1165	prb1 his4	Mut^+ His^- Protease deficient
SMD1163	pep4 prb1 his4	Mut^+ His^- Protease deficient

Table 2.2 Genotypes and phenotypes of some *P. pastoris* strains

Isolation of the *AOX*1 gene and its promoter (Ellis *et al*., 1985) and subsequently developed vectors, strains and molecular biology protocols resulted in a fully developed yeast expression system. The system relies on the integration of the introduced DNA into the yeast genome via homologous recombination (Cregg *et al*., 1985). The foreign DNA is first cloned in a bacterial host, commonly *E. coli*, using the integrative expression vectors. Subsequently isolated and linerized vectors recombinate via their free ends to a homologous region in the chromosome and the recombinant gene within the plasmid sequence integrates into the target genome (Ausubel *et al*., 1994). Although there are autonomous replicative plasmids, which can be transformed in *P. pastoris* by spheroplasting, they are of low copy number, unstable and invariably integrate at one or more of the choromosomal loci (Sreekrishna *et al*., 1997). Thus the chromosomal integration of linear plasmids are preferred.

The expression system is designed to work in both *E. coli* and *P. pastoris* thus the vectors developed contain sequences for bacterial origin of replication for replication and maintenance in bacterial hosts and selectable markers for both the hosts, the wild type *HIS4* gene and the bacterial kanamaycin/ ampicilin resistance genes (Romanos, 1995). However there are limitations of the system such as; applicability only to his4 auxotrophic hosts and the large size of the marker genes increasing the vector size decreasing vector stability and maintenance. The vector series pPICZ and pPICZα were designed to contain a dominant selectable marker, the *Sh ble* gene and unlike the above three genes, it functions in *E .coli, P. pastoris* and other yeasts and higher eukaryotes. The 375 bp *Sh ble* gene from *Streptoalloteichus hindustanus*, encodes for a 13.665 Da protein conferring resistance to the drug zeocin, stoichiometrically by binding and inactivation of the drug independent of strain or genotype of the host (Higgins and Cregg, 1998).

The gene of interest is inserted in the yeast genome by a single crossover type insertion. There are several vectors each carrying a foreign gene expression cassette. The most important component of the cassette is the promoter sequence, either of *AOX*1 gene or glyceraldehyde 3-phosphate dehydrogenase gene (*GAP*). The vectors, ie; pPICZ and pPICZα, carrying promoter sequence of the *AOX1* gene also contain the transcriptional termination (TT) sequence from the *P. pastoris AOX1* gene that directs

efficient 3' processing and polyadenylation of the mRNAs, following the MCS which allow insertion of the foreign gene. Homologous recombination event occurs between the genome and either of the two *AOX1* regions, promoter or TT resulting in the insertion of one or more copies of the vector upstream or downstream of the *AOX1* or aox1::*ARG4* genes depending on the genotype of host (Higgins and Cregg, 1998). After the recombination most of the wild type methanol utilization hosts will contain the expression cassette and the intact *AOX*1 gene, while some others will be disrupted in the *AOX*1 gene by the replacement of the cassette and the marker gene. These strains will have a Mut^s phenotype since they will utilize methanol by the transcriptionally weaker *AOX2* gene. These Mut^s strains can be identified by their slow/no growth on methanol medium, while growing normally on other carbon sources such as glucose or glycerol (Cereghino and Cregg, 2000).

P. pastoris cells are haploid and mating between cells occur in nitrogen limited medium. Complementary markers are available for use in mating assays; ADE1, ARG4, G418, HIS4, URA3 and Zeo^r. The diploids are stable unless they are subjected to nutritional stress (Cereghino and Cregg, 2000). The presence of multiple selectable markers allows coexpression of two or more proteins in the same strain (Vuorela *et al*., 1997).

The endogenous proteins of *P. pastoris* are secreted to extracellular medium at very low levels. Secretion of recombinant proteins, by the use of a secretion signal sequence is more favorable than intracellular expression considering that the recombinant proteins will compromise the vast majority of the extracellular protein increasing the rate of product recovery. However proteins that are not secreted by their native host may fail to be expressed correctly and secreted. There are two secretion signal sequences used; one is the *S. cerevisiae* α-factor prepro signal sequence and the other is *P. pastoris* acid phosphatase gene derived signal sequence, former being used with the most success. The vectors pPICZα contain the *S .cerevisiae* α-factor prepro signal sequence upstream of the MCS, which also includes the yeast consensus sequence. The consensus sequence is an ideal sequence for the interaction with its regulatory protein. Exact DNA sequence varies from gene to gene, depending on the specific consensus response elements that bind transcription factors that allow specific

control of gene expression, a promoter should, therefore, contain an element which is identical to or very close to the consensus sequence. There are two defined yeast consensus sequences, G/A NNATGG or A/V AA/TAATGTCT, either one should be included at the upstream of the foreign gene sequence to be expressed (Romanos *et al*. 1992).

The most important drawback of using a yeast expression system raises from the different glycosylation pattern from higher eukaryotes. Lower eukaryotes add O-linked oligosaccharides composed solely of mannose, whereas a variety of complex sugars are added in mammals. N-glycosylation is also different, mammalian Golgi apparatus performs in a way to generate high mannose type oligosaccharides, a mixture of several different sugars. The Golgi apparatus of yeast *S. cerevisiae*, on the other hand, elongates the N-linked core units through the addition of mannose outer chains and when a higher eukaryotic protein is processed in yeast this results in the phenomenon called hyperglycosylation. These hyperglycosylated recombinant proteins, with their long outer chains, are potential to be misfolded, unfunctional and antigenic for mammals. In *P. pastoris*, unlike *S. cerevisiae*, hyperglycosylation occur very rarely, but there are some examples of hyperglycosylated recombinant proteins. Also *P. pastoris* seems to be incapable of adding α 1,3-terminal mannose to oligosaccharides, another problem faced when using *S. cerevisiae* (Cereghino and Cregg, 2000, Higgins and Cregg, 1998). Some other problems can arise during use of *P. pastoris* as a host for foreign proteins, such as low recombination efficiencies of linear vector constructs to yeast genome, low expression levels due to toxicity of the foreign protein, failure to express AT-rich genes due to premature transcriptional termination (Romanos *et al*., 1992). proteolysis of secreted proteins and inefficient secretion of large proteins (Raymond *et al*., 1998).

3 MATERIALS AND METHODS

3.1 Materials

Details of materials used in this work are given below.

3.1.1 Chemicals

Chemicals that are used are listed in Appendix A.

3.1.2 Molecular biology kits

Molecular biology kits that are used for DNA isolation, gel extraction, DNA cleanup/desalting, yeast cloning /expression and protein analysis are listed in Appendix B.

3.1.3 Other materials

Details of materials including, cells, plasmids, DNA markers, protein markers, enzymes and enzyme buffers are listed in Appendix C. Maps of plasmids are given in Appendix E.

3.1.4 Equipment

Equipment that is used for general laboratory procedures are listed in Appendix D.

3.1.5 Primers

Primers were designed according to the coding sequence of *GPA*1 (NCBI accession number: AC004484) reported by Ma *et al*., (1990). Two different sets of primers were designed for amplification of two different products, *GPA1* and *GPA1*'. Forward primer for insertion of GPA1 into pPICZC vector included yeast consensus sequence at the beginning of the gene. In the reverse primers stop codon was not included in order to fuse the coding sequence with c-*myc* epitope and His-Tag. All primers used were synthesized by SEQLAB (Germany).

3.1.6 Buffers and solutions

Standard buffers and solutions used in cloning and molecular manipulations were prepared according to the protocols in Sambrook *et al*., 1989.

3.1.7 Buffer for agarose gel electrophoresis

1 X TAE (Tris-EDTA-Acetate) buffer was used for preparation of 1% and 0.8 % agarose gels. Unless otherwise stated 1% agarose gels were used. Gels were electrophorated at 100mV for 45 minutes. DNA was visualized by including 0.005% ethidium bromide in the gel during its preparation.

3.1.8 Buffer for SDS polyacrylamide gel electrophoresis

1X Tris-Glycine-SDS (sodium dodecyl sulfate) buffer was used for polyacrylamide gel electrophoresis. Gels were run at constant voltage , 60 mV, for about 2 hours.

3.1.9 Buffers for Western Blotting

Transfer buffer (Tris base, Glycine and methanol) was used in bloting the membrane. The membranes were blocked with blocking solution, 5% milk powder in PBS-Tween 20 (0,1%).

3.1.10 Culture medium

3.1.10.1 Liquid medium

3.1.10.1.1 Liquid medium for *E. coli*

LB Broth (Lenox L broth) from Sigma was used for liquid culture of bacteria. This mixture contains tryptone, yeast extract, and sodium chloride, which are mixed in appropriate amounts. 20 g of LB Broth was used for preparation of 1 L liquid medium. The pH of the medium was adjusted to 7.5 for growth of zeocin-resistant strains. The liquid medium was autoclaved at 121^0C for 20 min. before using. Kanamaycin at a final concentation of 50 μg/ml and zeocin at a final concentration of 25 μg/ml were added to liquid medium for selection.

3.1.10.1.2 Liquid medium for *Pichia pastoris*

YPD was used for liquid culture of yeast. The medium contains 1% yeast extract, 2% peptone and 2 % dextrose. Yeast extract and peptone was dissolved in water and autoclaved at 121^0 C for 20 min. Sterile dextrose was added before using. Zeocin , at a final concentration of 100 μg/ml was added when antibiotic selection is required.

BMGY, buffered complex glycerol medium, was used for biomass generation before induction of expression. This medium contains 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB (yeast nitrogen base), $4*10^{-5}$ % biotin and 1% glycerol. Yeast extract and peptone was dissolved in water and autoclaved at 121^0 C for 20 min. Other components were separately either autoclaved or filter sterilized and added to yeast extract and peptone mixture.

BMMY, buffered complex methanol medium, was used for expression of recombinant proteins. This medium is similar to BMGY, except there is 0,5 % methanol instead of 1% glycerol.

3.1.10.2 Solid medium

3.1.10.2.1 Solid medium for *E. coli*

LB Agar (Luria Bertani, Miller) from Sigma was used for preparation of solid medium for the growth of bacteria. This mixture contains tryptone, yeast extract, sodium chloride, and agar, which are mixed in appropriate amounts. 40 g of LB Agar was used for preparation of 1 L solid medium. The appropriate amount of LB Agar is dissolved in correspondent amount deionized water for autoclaving at 121^0 C for 20 min. Autoclaved medium was poured to petri plates (~20 ml/plate) after cooling down to room temparature. Kanamaycin at a final concentation of 50 μg/ml was added when antibiotic selection was required. 40 μl of 100mM IPTG and 40 μl of 40 mg/ml X-Gal were spread on plates for blue-white selection.

LSLB Agar was used for zeocin selection of bacteria on solid medium. This medium contains LB Broth (Lenox L broth) and Agar-agar. LB Broth contains tryptone, yeast extract, and sodium chloride, which are mixed in appropriate amounts. 20 g of LB Broth and 15 g of agar was used for preparation of 1 L solid medium. The pH of the medium was adjusted to 7.5 and the medium was autoclaved at 121^0 C for 20 min. After cooling down below 60^0 C, zeocin at a final concentration of 25 μg/ml was added and medium was poured to petri plates (~20 ml/plate).

3.1.10.2.2 Solid medium for *Pichia pastoris*

YPD agar was used for growth of yeast on solid media. The medium contains 1% yeast extract, 2% peptone, 2% agar-agar and 2 % dextrose. Yeast extract and peptone was dissolved in water , agar was added and autoclaved at 121^0 C for 20 min. After cooling down below 60^0 C sterile dextrose and zeocin at a final concentration of 100 μg/ml, when necessary, was added and medium was poured to petri plates (~20 ml/plate).

YPDS agar with zeocin was used for growth of either electroporated or Easycomp™ transformed yeast. The medium contains 1 M sorbitol with the components of YPD agar and prepared as described for YPD agar.

MDH, minimal dextrose medium with histidine, was used as a control for determination of Mut^+ phenotype. This medium contains 1.34% YNB, $4*10^{-5}$ % biotin , 2 % dextrose, 0.004 % histidine and agar-agar. Agar was dissolved in distilled water and autoclaved. After cooling down below 60^0 C separately autoclaved or filter sterilized components were added and medium was poured to petri plates (~20 ml/plate).

MMH, minimal methanol medium with histidine, was used to determine Mut^+ phenotype. This medium contains 1.34% YNB, $4*10^{-5}$ % biotin, 0.5 %methanol, 0.004 % histidine and agar-agar. Agar was dissolved in distilled water and autoclaved. After cooling down below 60^0 C separately autoclaved or filter sterilized components were added and medium was poured to petri plates (~20 ml/plate).

3.1.11 Sequencing

Sequencing service was commercially provided by SEQLAB (Germany).

3.2 Methods

3.2.1 Culture growth

3.2.1.1 Growth of *E. coli*

The *E. coli* cells were grown overnight (12-16h) at 37 °C shaking at 300 rpm in LB Broth (Lenox L broth) prior to any application. LB Agar (Miller's LB agar) and LSLB Agar solid media was used as unselective and selective solid medium for the growth of bacteria.

Protocols for liquid and solid culture growth and the other applications including competent cell preparation, glycerol stocks were done according to Sambrook *et al*.(1989).

3.2.1.2 Growth of *Pichia pastoris*

The Pichia pastoris cells were grown overnight at 30 °C shaking at 250 rpm in YPD medium for general purposes. Pichia pastoris strains X-33, GS115and KM71H were grown in BMGY for biomass generation before induction. BMMY medium was used for induction.

Liquid and solid culture growth and the other applications including competent cell preparation, glycerol stocks were carried out according to protocols described in manufacturer's manual (Invitrogen).

3.2.2 PCR

Reaction volumes and final concentrations of components for PCR were manufacturer's recommendations (Fermentas). Annealing temperatures of primers were estimated according to manufacturer instructions (Fermentas). pCIT 857(Ma *et al*., 1990) was used as template.

PCR amplification of GPA1 and GPA1' were carried out using the thermal cycle conditions given below

GPA1:

1. 94^{o}C…3 min.

2. 94^{o}C…1 min.
3. 60^{o}C…1 min. **30 cycles**
4. 72^{o}C…1 min.

5. 72^{o}C…1 min.

6. 4^{o}C…HOLD

*GPA*1':

1. 94° C…3 min.

2. 94° C…1 min.
3. 53° C…1 min. **30 cycles**
4. 72° C…1 min.

5. 72° C…1 min.

6. 4° C…HOLD

PCR products were purified directly with Quiaquick® PCR Purification Kit (250) (QIAGEN)

3.2.3 Subcloning

GPA1 and *GPA1'* were subcloned using pCR® II- TOPO® vector (Invitrogen) following the basic protocols recommended by the manufacturer. PCR amplified and purified *GPA*1 and *GPA*1' were ligated into pCR® II-TOPO® vector, reaction mixture was incubated at least 30 min. at room temperature (~25^0 C) and 1µl 6X TOPO® Cloning Stop Solution was added to stop the ligation reaction. Endonuclease deficient strains of *E. coli*-XL1 Blue, TOP10 and TOP10F' were transformed with ligation mixture and spread on both antibiotic(kanamaycin) and blue-white selective plates. Positive colonies were selected and grown in selective liquid media for both glycerol stock preparation and plasmid isolation. Where stated, the presence of the insert in bacteria was verified by direct colony PCR using the appropriate primers. Plasmid isolation was done either with Qiaprep® Spin Miniprep Kit (250) (QIAGEN) or

following to the alkaline lysis protocol from Sambrook *et al*.(1989). Purified plasmids containing *GPA1* and *GPA1*' were digested with appropriate restriction enzymes according to suppliers instructions to verify the presence of *GPA*1. (Enzyme/reaction mix) v/v ratio was kept at 1/10 or smaller in all digestions.

Purified plasmids and digested plasmids were analyzed by agarose gel electrophoresis. Appropriate DNA markers were used for size and concentration determination. In addition, concentration and $OD_{260/280}$ ratio were monitored by absorption measurements. Frozen stocks of *E. coli* containing pCR® II- TOPO® vector with *GPA*1 or *GPA*1' were prepared either using CYROBANK or in LB with 15% glycerol and kept at -80° C according to the protocol from Sambrook *et al*.(1989). QIAGEN® Plasmid Midi Kit (100) (QIAGEN) purified plasmids containing *GPA1*/*GPA1'*were sent for sequence analysis. Plasmids were checked by restriction and electrophoretic analysis before sequencing.

3.2.4 Directional cloning using expression vectors

3.2.4.1 Utilization of the subcloning construct

In this approach *GPA1* and *GPA1*' were digested out of the constructs pCRII-TOPO+GPA1 and pCRII-TOPO+GPA1' with appropriate enzymes, analyzed by agarose gel electrophoresis and DNA fragments corresponding to the genes were purified from the agarose gels with the view of insertion into expression vectors.

3.2.4.2 Direct insertion into expression vectors

PCR mixtures containing amplified GPA1 and GPA1' fragments were purified using Quiaquick® PCR Purification Kit (250) (QIAGEN) and digested with appropriate

enzymes. Similarly the expression vectors pPICZC and pPICZαB were digested with corresponding enzymes.

3.2.5.2.1Gel extraction method

The digestion mixtures were purified by agarose gel extraction using Quiaquick® Gel extraction Kit (250). *Eco*RI and *Xho*I double digested *GPA*1 and pPICZC were ligated in a 3:1 ratio. *Eco*RI and *Xba*I double digested *GPA*1' and pPICZαB were ligated in a 3:1 ratio. Ligation mixtures were incubated overnight at room temperature. After heat deactivation of the T4 DNA ligase at 60 °C, competent TOP10F' cells were transformed with ligation mixtures and spread on LSLB plates containing zeocin.

3.2.5.2.2 **Ethanol precipitation method**

The digested vector and fragment was mixed with 1/10 volume of 3 M sodium acetate, pH 5.2, and 2,5 times volume of 100% ethanol and DNA was precipitated overnight at –20 °C. After recovering the precipitate and washing with 80% ethanol, dried pellet was resuspended in sterile distilled water. Appropriate amount of 10X ligase buffer and T4 DNA ligase was added and reaction was incubated overnight at 16 °C.

After heat deactivation of the ligase, competent TOP10F' cells were transformed with ligation mixtures. Transformants were spread on LSLB plates containing zeocin and incubated at 37 C° for overnight. Positive colonies were selected and colony PCR was performed for the initial verification of the insert. Colonies from which *GPA*1 and *GPA*1' were amplified were grown overnight in liquid media containing zeocin for long term storage and plasmid isolation. Isolated plasmids were sequenced and digested with appropriate restriction enzymes for the verification of the insert.

3.2.5 Transformation of *Pichia pastoris*

Three different methods were applied to recombinate the linear vector DNA into yeast genome. The yeast strains, GS115 and KM71H, were prepared and transformed as described in manufacturer's manual (Invitrogen) and vector DNA was prepared as described below for all the methods.

3.2.5.1 Preparation of the insert

The sequence verified constructs were isolated from *E. coli* TOP10F' cells by QIAGEN® Plasmid Maxi Kit (500) (QIAGEN). The unmodified vectors were isolated from *E. coli* TOP10F' cells by QIAGEN® Plasmid Midi Kit (100) (QIAGEN). The plasmids were linearized with either one of the restriction enzymes, *Bst*XI and *Sac*I. *Bst*XI linearization reaction was held at 55°C, whereas *Sac*I reaction was incubated at 37 °C and both reactions were stopped by heat deactivation of the enzyme The linearized plasmids were phenol:chloroform:isoamyl alcohol extracted as described in Ausubel *et al*.,(1994) for use in electroporation. Linearized/phenol extracted vectors were ethanol precipitated. The final DNA concentration and volume varies with the transformation method applied as given in table 3.1

Method	[DNA]	medium	incubation	Details
Easycomp™ Transformation	3 µg in 5 µl	YPDS+zeocin	3-10 days,30°C	-
Electroporation	5-10 µg in 5µl	YPDS+zeocin	3-10 days,30°C	3.2.5.2
Lithium Chloride Transformation	5-10 µg in 50 µl	YPD+zeocin	3 days,30°C	3.2.5.3

Table 3.1Summary of yeast transformation methods

3.2.5.2 Electroporation

40 µl of cells were mixed with 5 µl of linear vector DNA and transferred to 0.2 cm electroporation cuvettes. Electroporation was performed using BTX ECM 630 with the conditions; given in table 3.2

Voltage	1500 V
Capacitor	0025 µF
Resistor	125 Ω
Pulse	3,8 ms

Table 3.2 Electroporation device parameters.

3.2.5.3 Lithium chloride transformation

For the homologous recombination to occur a carrier DNA must be included in the transformation mixture in lithium chloride method. 2 mg/ml denatured, fragmented salmon sperm DNA was used as carrier DNA. Double stranded DNA from salmon testes was dissolved, fragmented and denatured as described in Ausubel *et al*., (1994). Sonication was done in order to fragment the DNA. Size distribution of sonicated DNA was analyzed on 0.8% agarose gel and optimum sonication time was determined. After optimum size distribution was achieved, phenol:chloroform extraction and ethanol precipitation was done. Fragmented DNA was boiled in order to obtain single stranded DNA.

3.2.6 Yeast colony PCR

Positive transformed yeast colonies were selected and presence of *GPA*1 or *GPA*1' was verified by colony PCR using 5' AOX and 3' AOX sequencing primers. These primers amplify AOX1 promoter region of the *P. pastoris* genome, and also the

gene inserted within the 5' AOX and 3' AOX priming sites, integrated into genome. Colony PCR was performed as described in Burke *et al*.,(2000).

3.2.7 Verifiying the Mut$^+$ phenotype

PCR verifed GS115 colonies were streak on both MDH and MMH plates. After 2 days incubation at 30 °C, plates were analyzed for their methanol utilization phenotype by comparing their growth with growth of control strains. Mut$^+$ control strain, GS115/pPICZC/*lacZ* shows normal growth on both plates, whereas control strain GS115 Albumin is Mut^S and shows little or no growth on MMH plate.

3.2.8 Expression

The PCR verified KM71H and Mut$^+$ GS115 integrants were induced as described in manufacturer's manual (Invitrogen)**.** Cells were grown in BMGY medium until the culture reaches an OD_{600} of approximately 2-6. Harvested cells were resuspended in BMMY medium and 0.5% methanol was added every 24 hours to maintain induction. KM71H integrants are Mut^S showing slow growth on methanol, thus they were grown in higher volumes of BMGY and smaller volumes of BMMY than the Mut$^+$ GS115 integrants. Although baffled flasks are recommended for expression, normal shake flasks were used and cells were grown at 28-30 °C at 250 rpm. Control strains were induced to test the effectiveness of expression conditions. GS115 Albumin was induced as a control for testing expression of KM71H, which is Mut^S and secretes albumin to the medium, whereas for GS115 integrants the Mut$^+$ strain GS115/pPICZC/*lacZ* ,which intracellulary expresses β-galactosidase, was used as a control. In order to analyze the background intracellular expression, strains transformed with the unmodified vector, pPICZC were also induced. At defined time points samples were taken from the cultures for absorption measurements, SDS-PAGE and Western Blot analyses. In order to analyze intracellulary expressed proteins cells were lysed using 0.5 mm acid washed glass beads as described in manufacturer's manual (Invitrogen)**,** whereas for detection

of extracellular expression samples were pelleted and supernatants containing secreted soluble proteins were stored for analysis.

3.2.9 Western blotting

10% SDS-polyacrylamide gel was blotted on PVDF membrane at 25 mA constant current for 2 hours. Blotted membrane was blocked with blocking solution on orbital shaker for 1 hour at room temperature and incubated with anti-*myc*-HRP antibody for 1 hour at room temperature. The membrane was treated with ECL Advance Western Blotting Detection Kit (Amersham Biosciences) and resulting signals were analyzed by Hyperfilm ECL (Amersham Biosciences).

4 RESULTS

4.1 PCR Amplification of *GPA*1

4.1.1 Template Isolation

PCIT 857 is the original construct containing the *A. thaliana* G protein α subunit (Ma *et al.,* 1990, Bakkal, 2003). PCIT 857 was isolated from *E. coli* XL1Blue cells to be used as template in PCRs.

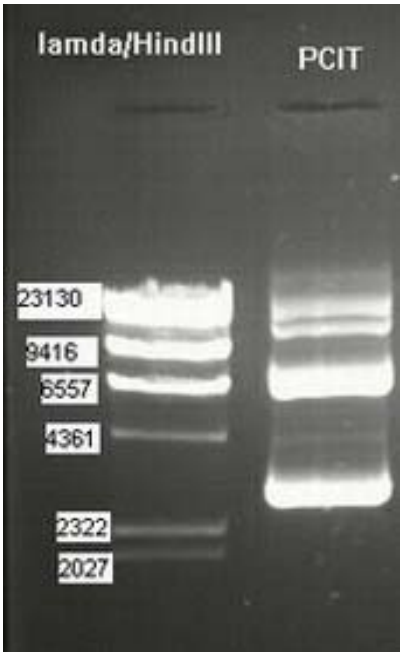


Figure 4.1 Analysis of isolated pCIT 857.

4.1.2 PCR amplification of target genes

*GPA*1 was amplified by PCR for subcloning using pCR® II- TOPO® and two different yeast expression vectors. *GPA*1 was cloned using pPICZC and *GPA*1' was cloned using pPICZαB.

4.1.2.1 PCR amplification of *GPA1*

GPA1 was amplified using primers designed with *Eco*RI and *Xho*I restriction enzyme sites;

Forward primer (GPA1-FP): 5'-ATA CCA GAATTC **GTC ATG G**GC TTA C-3'

Reverse primer (GPA1-RP): 5'-TAT ATA CTCGAG TAA AAG GCC AGC C-3'

The *GPA1* fragment amplified using GPA1-FP and GPA1-RP was subcloned using pCR® II- TOPO® vector and also cloned directly into pPICZC vector. Restriction enzyme sites are underlined and yeast consensus sequence is shown in bold letters.

PCR conditions were basically as described in section 3.2.2. Optimization of conditions with respect to primers was investigated at 0.5 μM and 1 μM final concentrations. As can be seen from the agarose gel analysis given in figure 4.2, the higher primer concentration resulted in efficient amplification of *GPA*1 by PCR using intact pCIT as template. The amplified PCR product is ~1173 bp, since the enzyme sites and yeast consensus sequence included in primers are added during amplification.

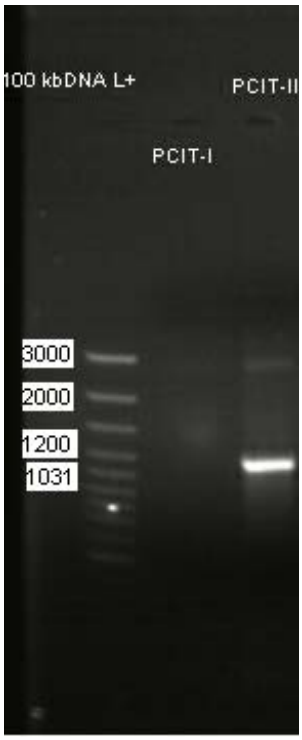


Figure 4.2 Analysis of PCR carried out at 0.5 μM (pCIT-I) and 1 μM (pCIT-II) final primer concentrations.~1173 bp fragment is seen in the lane labeled pCIT-II.

4.1.2.2 PCR amplification of *GPA*1'

*GPA*1' was amplified using primers designed with *Eco*RI in the forward and *Xba*I restriction enzyme site in the reverse primer.

Forward primer (GPA1'-FP):5'-GCG TC GAATTC **CC** ATG GGC TTA CTC TG-3'

Reverse primer (GPA1'-RP):5'-GAC GC TCTAGA **CC** TAA AAG GCC AGC CT-3'

The *GPA*1' fragment amplified using GPA1'-FP and GPA1'-RP was subcloned using pCR® II- TOPO® vector and also cloned directly using pPICZαB vector. Restriction enzyme sites are underlined and extra bases, added are shown in bold letters. Extra bases are added to bring *GPA*1 in frame with *S. cerevisiae* α factor prepro peptide and his-tag, in the upstream and downstream respectively of MCS of pPICZαB vector. PCR conditions were basically as described in section 3.2.2. The PCR product GPA1' is

~1172 bp since the enzyme sites and extra bases included in primers are added during amplification (figure 4.3).

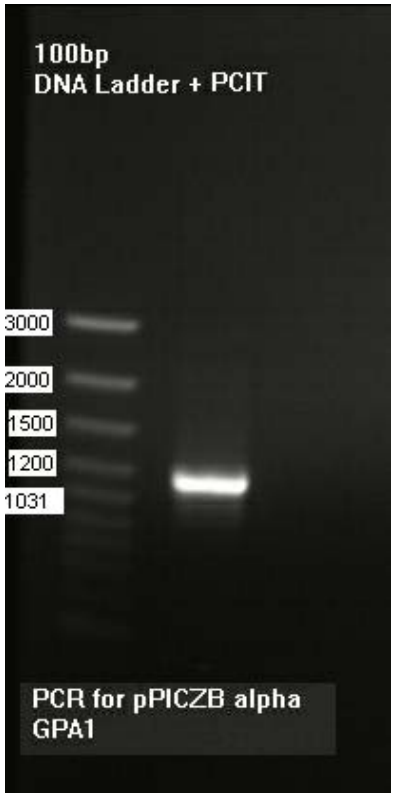


Figure 4.3 Analysis of PCR products using primers GPA1'-FP and GPA1'-RP, yielding a 1172 bp fragment.

4.2 Subcloning and Sequence Verification of *GPA1*

4.2.1 Insertion into pCR-II TOPO vector

For sequence verification all amplified *GPA*1 and *GPA*1' fragments were first inserted into pCR-II TOPO vector and TOP10F' cells were transformed with the construct. pCR-II TOPO vector facilitates A-tailing ligation of the fragment between two flanking *Eco*RI sites via topoisomerase reaction and presence of the insert can be verified by digestion of the isolated plasmids with *Eco*RI. Colonies were selected by

blue-white screening and plasmids isolated from liquid cultures grown from the colonies are shown in figure 4.4. Results of digestion revealing approximately 1170 bp fragments can be seen in figure 4.5. Clones TOPO+GPA-2 and TOPO+GPA-3 were sequenced for verification of the presence of *GPA*1 and the yeast consensus sequence. Clone TOPO+GPA1'-3 was sequenced for verification of the presence of *GPA*1' in the pCR-II TOPO vector. Alignment results of constructs with GPA1 and GPA1' are given in Appendix F.

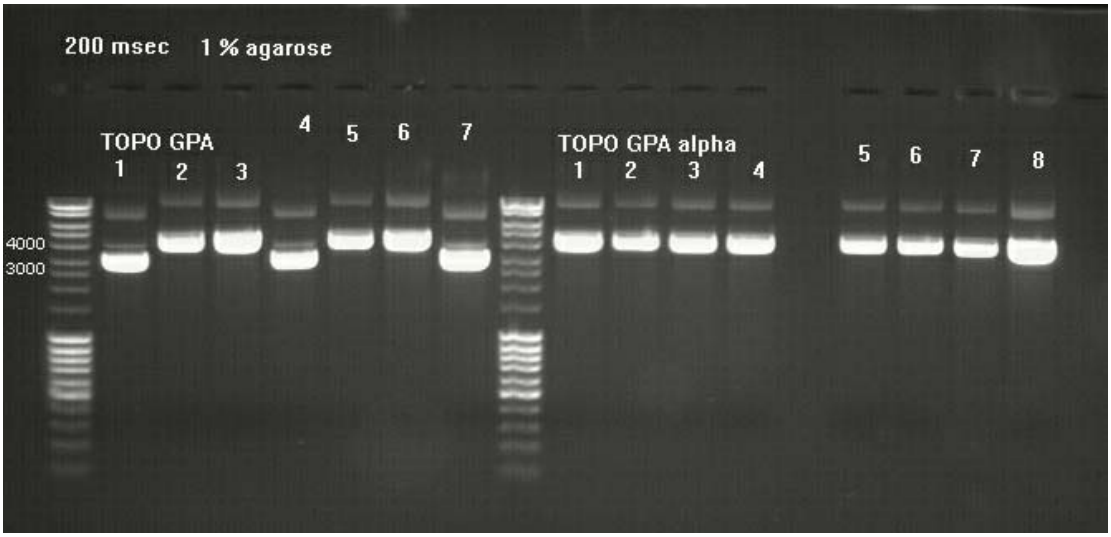


Figure 4.4 Analysis of plasmids isolated from colonies of PCR II TOPO constructs.

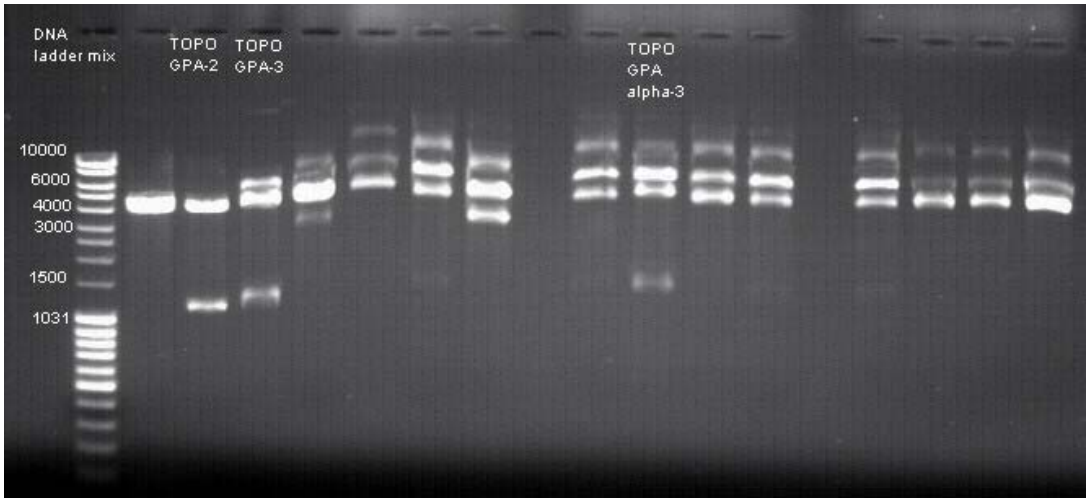


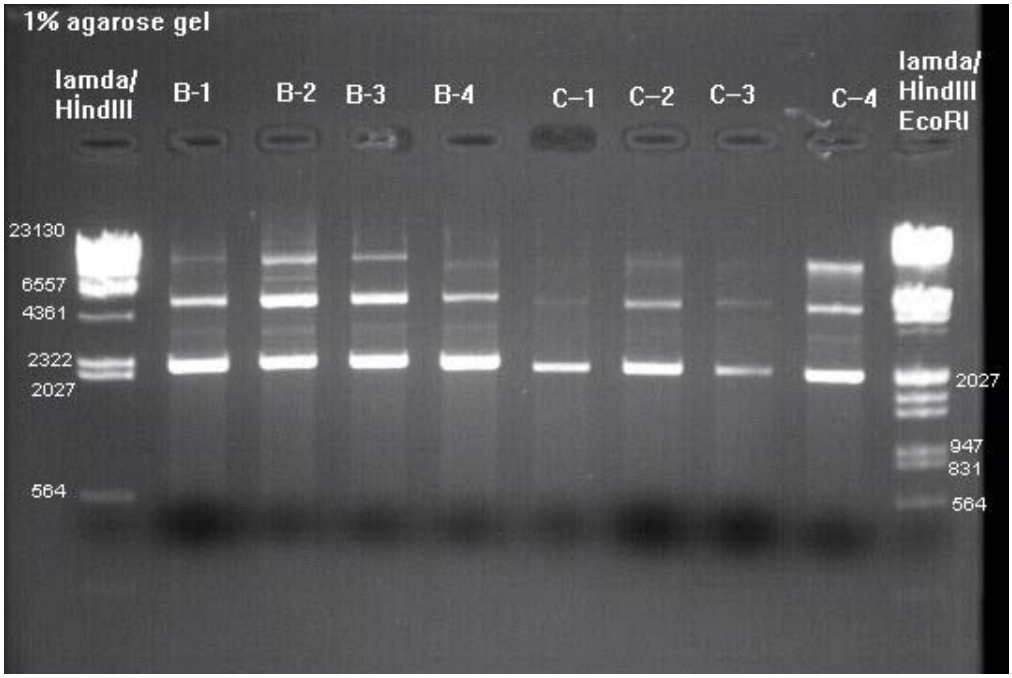
Figure 4.5 Analysis of *Eco*RI restriction enzyme digestion of plasmids isolated from colonies.

4.3 Cloning of *GPA*1 Using Expression Vectors

General strategy followed for cloning using expression vectors was digestion of the *GPA*1 and *GPA*1' fragments out of the subcloning constructs using the appropriate restriction enzymes followed by ligation with the expression vector prepared by digestion with the same enzymes. However, digestion out of the subcloning constructs did not yield enough fragment for subsequent ligation reactions (figure not shown) and in subsequent attempts PCR products were directly digested with the appropriate restriction enzymes (figure not shown). Digested vectors purified by gel extraction were ligated with digested fragments and were introduced into TOP10F' cells. Transformed cells were spread on LSLB plates containing zeocin. However, again, it was not possible to obtain colonies with this method. These negative results prompted us to use an alternative method for the ligation procedure as reported below.

Typical results of agarose gel analyses of purified and double digested cloning vectors are shown in figures 4.6 A and B respectively. The agarose gel analysis of vector isolation and double digestion steps performed in other ligation experiments will not be given as the results are identical to those shown in figure 4.6.

A



B

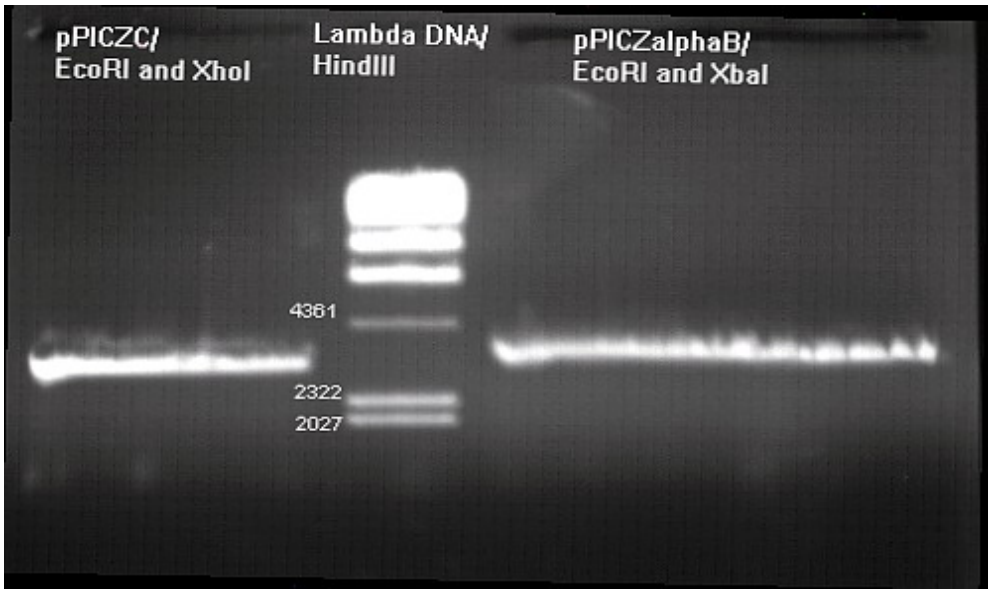


Figure 4.6 Analysis of isolation of expression vectors from the host *E. coli* TOP10F' cells.(A) and the result of double digestion reaction of purified expression vectors.(B)

4.3.1 Cloning of pPICZC+GPA1

Since the classical cloning approach did not work another method was followed. Here the digested vector was ethanol precipitated together with the digested insert for further purification as well as for resuspending the two components together in a small volume. Ligation reaction was carried out in this small volume. Afterwards the ligation mixture was introduced in *E. coli* TOP10F' cells which yielded many colonies on selective plates. As an initial screening colony PCR was carried out with gene-specific primers. Results given in figure 4.7 show that PCR of colonies 2, 5, 8, 9 and 10 resulted in the 1173 bp fragment likely to correspond to *GPA*1. Colonies 8, 9 and 10, on the other hand, gave rise to significant amplification of other sequences with *GPA*1 primers. In order to investigate the reason of this unspecific amplification, untransformed *E. coli* TOP10F' cells were used as template in a PCR with the *GPA*1 primers. The result of the reaction confirmed that these two genes are amplified also in the untransformed host (results not shown).

Figure 4.7 Analysis of PCR amplification results of colonies transformed with pPICZC+GPA1.

Selected colonies were cultured in liquid medium for plasmid isolation, results of which are shown in figure 4.8. Plasmids isolated from colonies # 2, 4, 8 and 10 migrate to a position ~1000bp higher than pPICZC, indicating the presence of insert. Plasmid isolation from colony-5 (which gave positive results in the PCR screening) failed. Subsequent digestion of plasmids with EcoRI and XhoI verified the presence of *GPA*1, figure 4.9, and clones pPICZC+GPA1-2, -4, -8 and –10 were sent for sequencing. Alignment of clone pPICZC+GPA1-2 with *GPA1* starting with the yeast consensus sequence including all sequences between *Xho*I site of the insert and stop codon of poly-histidine tag of the vector is shown in Appendix F.

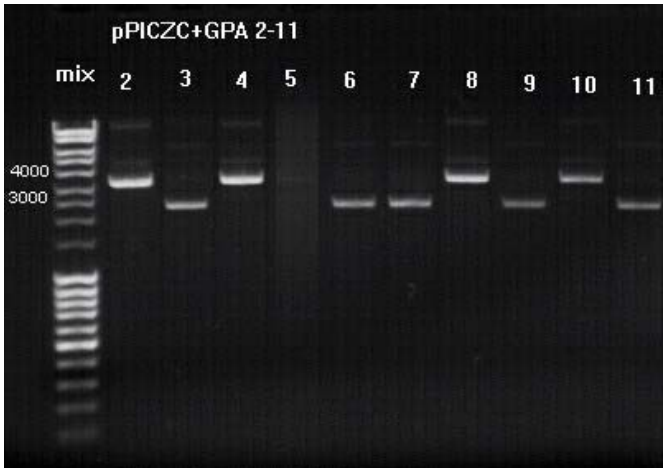


Figure 4.8 Analysis of plasmid isolation from selected colonies transformed with pPICZC+GPA1.

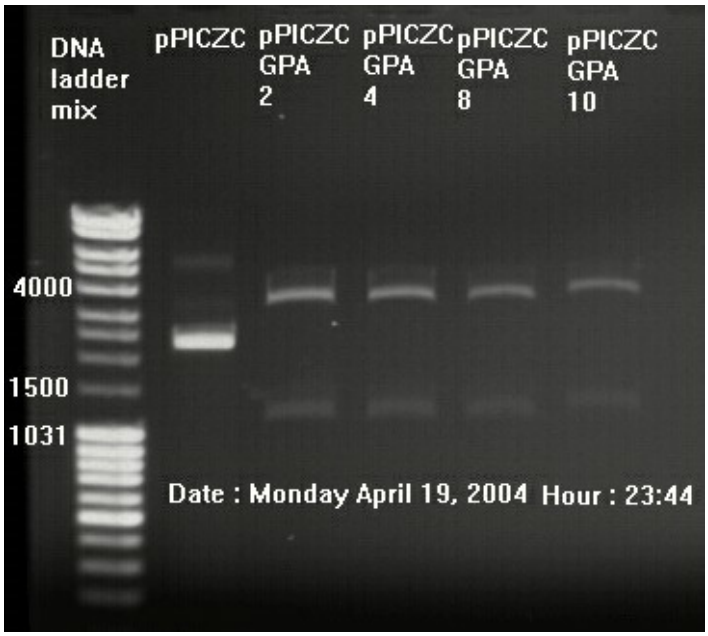


Figure 4.9 Analysis of EcoRI and XhoI double digested constructs for verification of the presence of *GPA*1. The plamids pPICZC+GPA–2, 4, 8, 10 have the fragment of GPA1 size, ~1151 bp.

4.3.2 Cloning of pPICZαB+GPA1'

The cloning of *GPA*1' fragment using the expression vector pPICZαB was performed as described above and results of colony PCR are presented in figure 4.10. Cells from the colonies yielding a 1172 bp fragment upon amplification were cultured for plasmid isolation and double digestion with *Eco*RI and *Xba*I. As shown in figure 4.11 colonies 2 and 3 contained the constructs carrying *GPA*1'. Plasmids isolated from these, pPICZαB+GPA1'-2 and –3, were sent for sequencing. Alignment of clone pPICZαB+GPA1'-2 with *GPA1* starting with α-factor signal sequence including all sequences between *Xba*I site of the insert and stop codon of poly-histidine tag of the vector is shown in Appendix F.

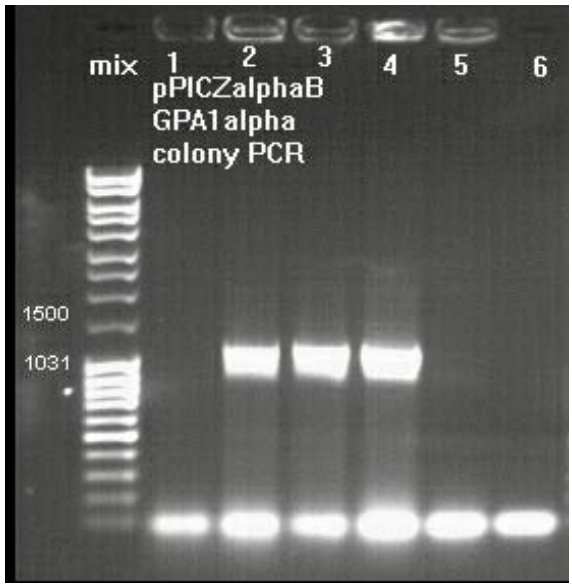


Figure 4.10 Colony PCR results of selected colonies after transformation with pPICZαB+GPA1' construct

Figure 4.11 *Eco*RI and *Xba*I double digestion of isolated plasmids. The uncut plasmids are also included in the analysis for reference. The band seen at ~1152 bp corresponds to *GPA*1'

4.4 Transformation of *Pichia pastoris*

Three different methods were used for the transformation of *P. pastoris*; Easycomp™ Transformation, electroporation and lithium chloride transformation. All three approaches require large amounts of linearized insert DNA for recombination into the yeast genome. For this purpose sequence verified constructs and the parent vectors were isolated from *E. coli* TOP10F' cells using large-scale protocols.

4.4.1 Preparation of the insert

The DNA concentrations of isolated plasmids and the method used are given in table 4.1. Complete linearization is required for the integration of 5' AOX region of the plasmid at the AOX1 promoter of *P. pastoris*. Optimization studies of the linearization reaction showed that reaction could not be completed in 3 hours or overnight for either of the enzymes *BstX*I or *Sac*I; some undigested construct could be detected as seen in figure 4.12. When compared, reaction with *Sac*I resulted in less amount of undigested material for both 3 hours and overnight incubation and longer incubation time was selected. During all transformation procedures plasmids were linearized overnight by *Sac*I and a typical result of the reaction is shown in figure 4.13

plasmid	concentration (μg/μl)	isolation method
pPICZC+GPA1-2	1,71	Maxi prep
pPICZαB+GPA1'-2	2,52	Maxi prep
pPICZC	1,445	Midi prep
pPICZαB	0,84	Midi prep

Table 4.1 Concentrations of isolated plasmids calculated from absorbance measurements performed at 260 nm.

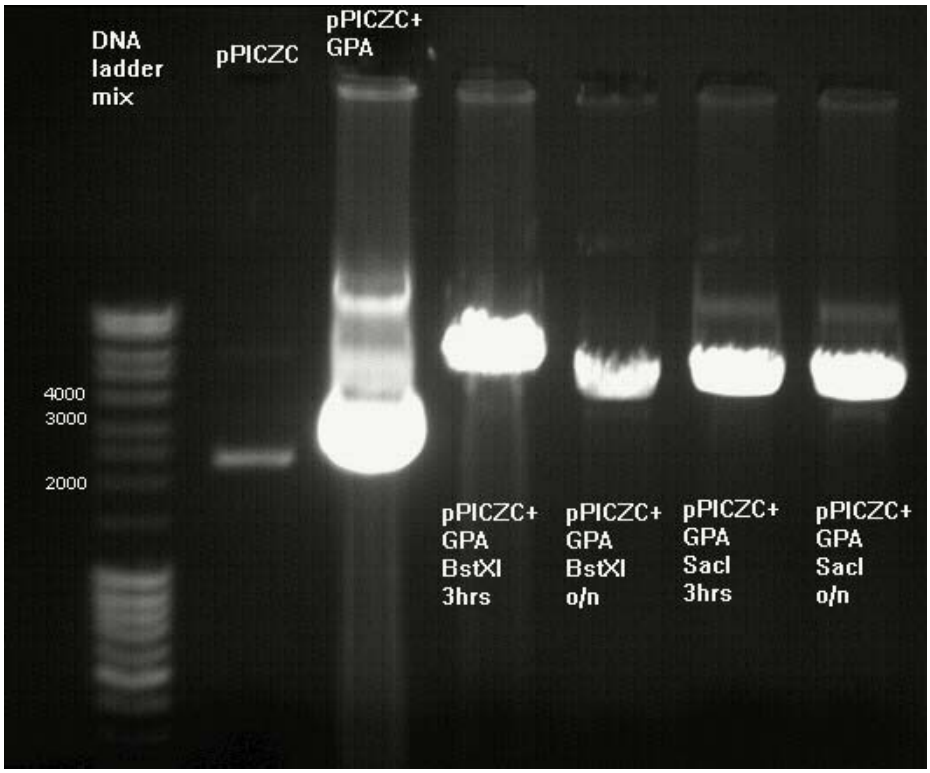


Figure 4.12 Analysis of *Bst*XI and *Sac*I linerization reactions of construct pPICZC+GPA1-2 at different reaction times. Samples were overloaded in order to visualize the remaining undigested material

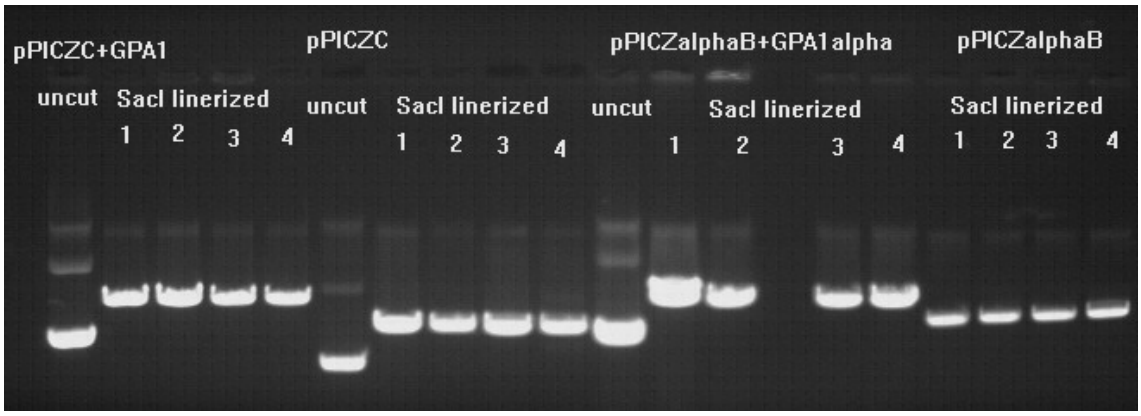


Figure 4.13 Analysis of *Sac*I digestion of constructs and corresponding original (uncut) vectors. The unlinearized plasmids are included in order to check the efficiency of the reaction.

4.4.2 Preparation of carrier DNA for lithium chloride transformation

Transformation of yeast using an alkali cation is a common procedure, and lithium acetate is used for *S. cerevisiae*. Lithium chloride transformation is a modified version of lithium acetate since latter alkali cation is not effective with *P. pastoris*. Transformation with either method requires the presence of a carrier DNA in the transformation mixture. Carrier (salmon sperm) DNA must be fragmented and denatured into single stranded form. Dissolved salmon sperm DNA was sonicated in order to achieve fragmentation. The longer the fragments, the higher the transformation efficieny, but longer fragment size would result in a more viscous and unwieldy solution. The optimum size distribution for fragments ranges from 2 kb to 15 kb with a mean size of 7 kb. Figures 4.14 and 4.15 show the results of sonication of the salmon sperm DNA as a function of time. First attempts resulted in over fragmentation of the DNA, even for 8 seconds of sonication (Figure 4.14). In a second trial only 4 seconds of sonication was performed. This resulted in a better fragment size distribution (Figure 4.15), however, still yielding fragments smaller than the optimum size.

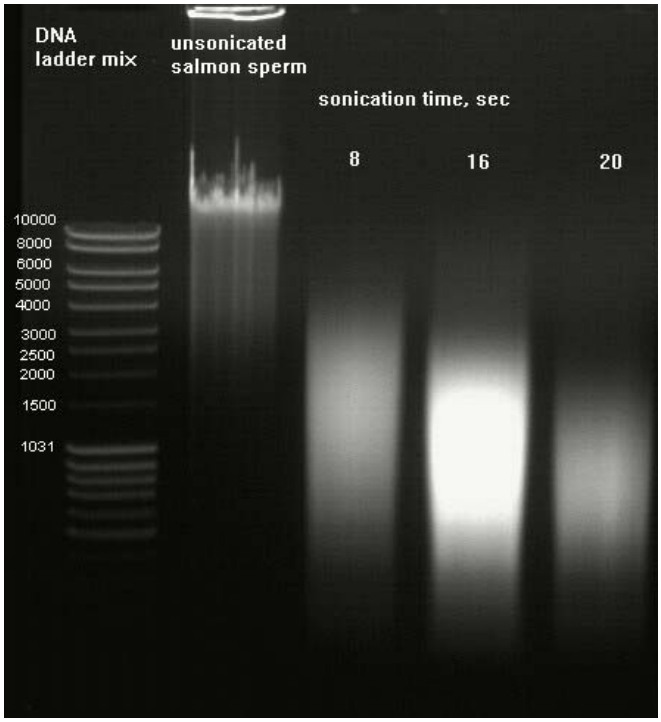


Figure 4.14 Size distribution of carrier DNA after sonication 8, 16 and 20 seconds. Analysis was carried out on a 0.8% agarose gel.

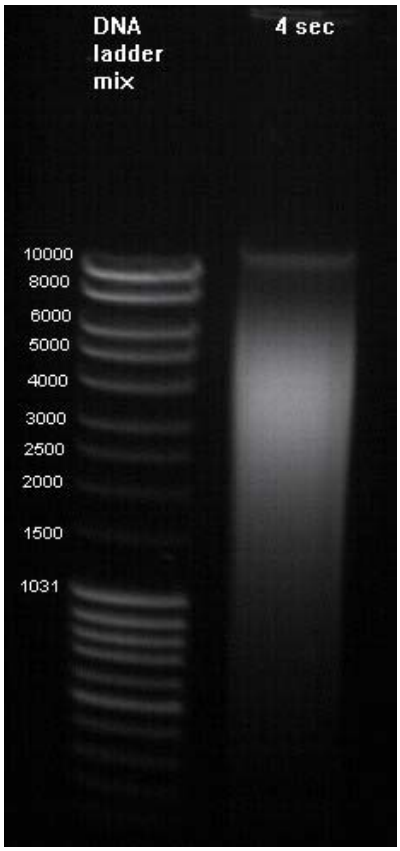


Figure 4.15 Size distribution of carrier DNA after sonication for 4 seconds , analyzed on a 0.8% agarose gel. The fragment size distribution is between 2 kb and 10 kb with a mean size of ~5kb.

4.4.3 Transformation

Results of transformations using lithium chloride and electroporation methods that gave colonies on selective plates are listed in table 4.2. The transformation of GS115 strain was more efficient than that of KM71H.

Name	number of colonies	insert	strain	Transformation method
7e	2	*GPA*1'	GS115	electroporation
10e	2	pPICZC	GS115	electroporation
1	5	*GPA*1	GS115	LiCl
5	1	*GPA*1’	GS115	LiCl
9	1	pPICZC	GS115	LiCl
1'	25	*GPA*1	GS115	LiCl
5'	2	*GPA*1’	GS115	LiCl
9'	1	pPICZC	GS115	LiCl
3	1	*GPA*1	KM71H	LiCl
7	2	*GPA*1’	KM71H	LiCl
11'	10	pPICZC	KM71H	LiCl

Table 4.2 List of colonies grown on selective plates.

4.4.4 Verification of insert by PCR amplification

Positive colonies were selected on the basis of colony PCR that verified the insert. PCR amplification of integrants with 5’ AOX and 3’ AOX primers, as described in section 3.2.6, is expected to result in two bands, one corresponding to size of the inserted gene and the other to the *AOX*1 gene which is approximatey 2.2 kb for strain GS115. For the KM71H integrants the size of this PCR product is 3.6 kb due to *ARG*4 insert in *AOX*1. Parent plasmids add extensions to the gene inserted as given in table 4.3

Vector	Added fragment length	
pPICZC	324 bp	
pPICZαB	592 bp	

Table 4.3 The size (in bps) of DNA fragments added to PCR products by the parent vectors. (manufacturer’s manual(Invitrogen)).

GPA1 gene is inserted into pPICZC expression vector, thus the expected PCR product is 1150 bp+324 bp, approximately 1500 bp long. *GPA*1' gene is inserted to pPICZαB expression vector resulting in a PCR product of size 1150 bp + 592 bp, approximately 1800 bp. Figures 4.16 and 4.17 show the results of colony PCR.

In figure 4.16 the first five lanes after the marker lane represent different colonies of transformant 1. Except colony 1-4 , the 2.2 kb *AOX*1 gene and 1.5 kb *GPA*1 gene were amplified, showing that the *GPA*1 gene is integrated into yeast genome without disrupting the *AOX*1 gene. The transformant 5 encodes for *GPA*1', as seen from the intense 1.8 kb band and, although very weak, the 2.2 kb band is also present. The 2.2 kb band is not seen with 9; the parent vector transformed GS115. The 3.6 kb region was not amplified for any of the KM71H transformants, although the foreign gene between the priming sites are amplified and products are 1.5 kb (*GPA*1) for transformant 3, and 1.8 kb for transformants 7-1 and 7-2 (*GPA*1'). Finally the PCR results of colonies obtained by *GPA*1'integration via electroporation are shown in lanes 7e-1 and 7e-2, the latter show, albeit weakly, the amplified *GPA*1' band of size 1.8 kb.

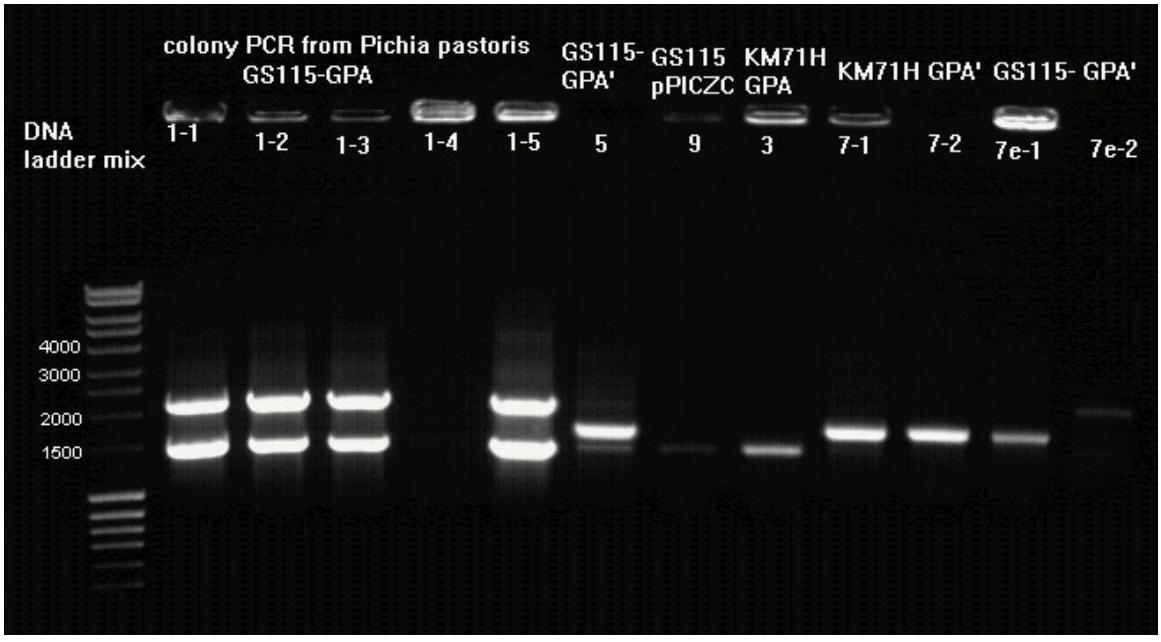


Figure 4.16 Analysis of colony PCR results. Templates used are named as indicated in table 4.2.

In figure 4.17 the first three lanes after the marker lane are colony PCR products of negative control strains. 2.2 kb *AOX*1 gene is amplified as expected from colonies of GS115/pPICZC/*lac*Z and GS115. The absence of the 3.6 kb band shows that the genomic DNA of strain KM71H was not amplified by the 5' AOX and 3' AOX primers.

Another interesting result is the 1.5 kb sized PCR product of GS115/pPICZC/*lac*Z, which is also present in lanes of figure 4.16 representing colonies from transformation with *GPA*1' or the parent vector pPICZC. It appears that this 1.5 kb band results from an unspecific amplification of a sequence in the parent vectors. But since the intensity of this ghost band is much lower than those of *GPA*1 transformants, it can be neglected in considerations of verification of *GPA*1.

PCR products arising from colonies of cells transformed with pPICZC are shown in lanes labeled as 9, 10e-1, 10e-2, 9', 11'-1, 11'-2 and 11'-3 in figure 4.17 the 2.2 kb band resulting from *AOX*1 gene is present in none of these lanes. But for lanes 9 and 10e-2 the 1.5 kb band is present, which may be correlated with the insertion of the parent vector as discussed above.

Lanes 1'-1, 1'-2 and 1'-3 show PCR products of colonies from 1', the first two containing the 2.2 kb and 1.5 kb bands verifying the presence of GPA1. The PCR products of colony 1-4, although the 2.2 kb band corresponding to *AOX1* gene is not very clear, display a very strong band at 1.5 kb resulting from amplification of *GPA*1.The last two lanes are PCR products of colonies transformed with *GPA*1', the one designated as 5'-1 with the corresponding band 1.8 kb, verifying the integration of *GPA*1' to the genome.

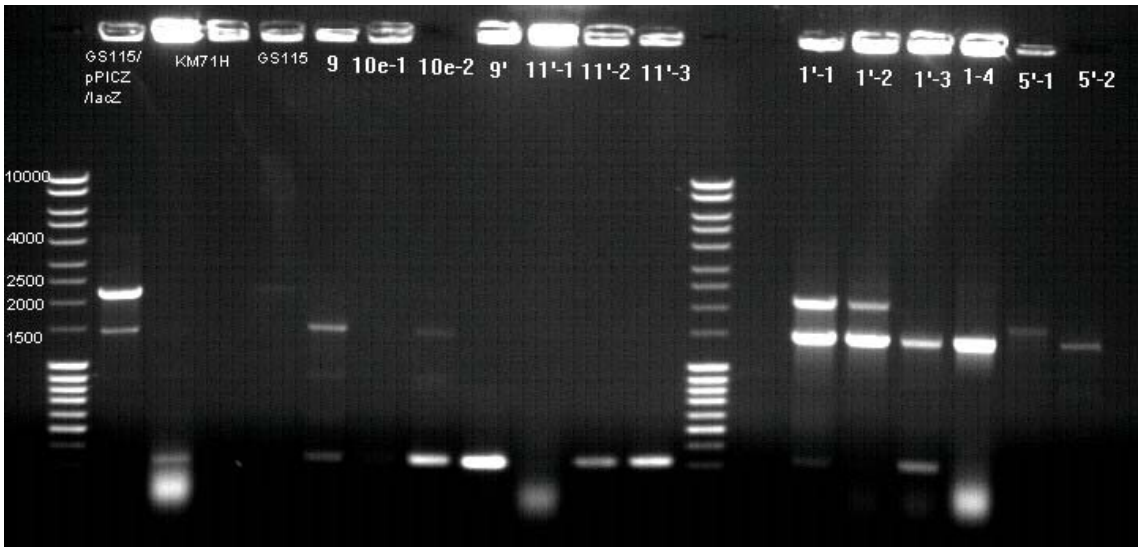


Figure 4.17 Analysis of colony PCR results. Templates used are named as indicated in table 4.2.

4.4.5 Determining the Mut$^+$ phenotype.

GS115 integrants were plated on both MDH and MMH plates in order to determine the Mut$^+$ phenotype. Mut$^+$ colonies showed normal growth on both plates verifiying that the *AOX*1 locus is intact, whereas Mut^S colonies showed little/no growth. Results are listed in table 4.4. Mut^S phenotype results from recombination events occurring in the 3' *AOX*1 region disrupting the wild-type *AOX*1 gene.

Phenotype	insert	colony #
Mut$^+$	*GPA*1	1-1-1-5, 1'-2
	*GPA*1'	7e-2, 5'-1
	pPICZC	9', 10e-2
Mut^S	*GPA*1	1'-1
	*GPA*1'	5'-2
	pPICZC	9

Table 4.4 Results of determination of Mut phenotype for GS115 integrants, growth of colonies were compared to those of the control strains, Mut$^+$ GS115/pPICZC/*lac*Z and Mut^S GS115 Albumin.

4.5 Expression

4.5.1 Induction of KM71H integrants

Colonies 3, 7-2, 11'-1(control for background native proteins) and GS115 Albumin (control of expression) were grown on glycerol for biomass generation for 20 hours. The OD_{600} measurements for determining cell growth are given in Appendix H.1. Yeast cells were induced with methanol at the exponential phase of growth, corresponding to an OD_{600} in the range 2 to 6 and samples were taken at different time points after induction for 48 hours. The control strain GS115 Albumin is expected to secrete albumin, molecular weight of 67 kDa, to the extracellular medium. After 48 hours on methanol as the only carbon source, cell lysates and extracellular medium concentrates were analysed by SDS-PAGE as shown in figure 4. 18. Secreted albumin is indicated by the arrow. Lysates of the same cells are electrophoresed in the lane labeled GS115 Albumin. The expected size of the recombinant Gα is about 46 kDa including *myc*-epitope and his-tag fusions (approximately 2.5 kDa). In figure 4.18 a band near 46 kDa is seen for the extracellulary expressed Gα (lane GPA1' secreted, lane GPA1' represents the cell lysate of the same sample), but a similar sized protein band is also seen in the lane GS115 Albumin secreted indicating that this protein is likely to be a native protein of the yeast.

As far as the intracellularly expressed recombinant GPA1 is concerned (lane GPA1) this gel could not be used to verify expression due to overloading of the endogenous proteins. Due to time limitations this experiment could not be repeated.

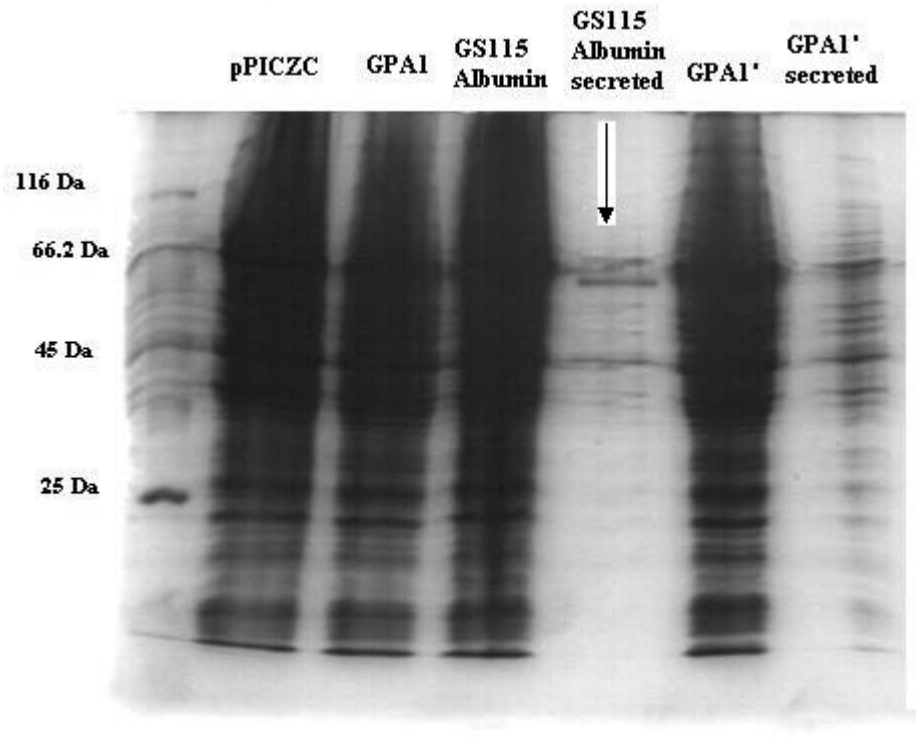


Figure 4.18 Analysis of induction of KM71H integrants by 12% SDS-PAGE , after 48 hours of induction

4.5.2 Induction of GS115 integrants

Several GS115 integrant strains were induced in order to screen for the optimized recombinant protein production. During each induction GS115/pPICZC/*lacZ* (labeled as lacZ on figures) was also included as the control of expression. This strain expresses intracellulary β-galactosidase, (MW: 119 kDa) as a *myc*-epitope fusion. Parent vector transformed GS115 cells (labeled as pPICZC on figures) were also induced to monitor the background protein levels. Yeast cells were induced with methanol at the exponential phase of growth, corresponding to an OD_{600} between 2-6. Samples taken at different time points during induction were analyzed by SDS-PAGE and Western Blotting.

4.5.2.1 Induction of GPA1' expression

Induction results for the GPA1' encoding strain as well as the above mentioned controls were analyzed on 10% SDS-PAGE and by Western blot using anti-*myc* antibody. The β-galactosidase (119 kDa) expression not detected after 6 hours of induction could be visualized as a weak band after 36 hours, indicated with the arrow on Figure 4.19, and as induction period increased (60 hours and 72 hours) higher amounts were detected (Figure 4.20). This result shows that we could detect expression of recombinant protein β-galactosidase after 36 hours of induction with methanol, but no secreted GPA1' could be visualized. A similar result is observed after 60 hours of induction. For confirmation of this result Western blotting using anti-*myc*-HRP antibody was carried out. As mentioned before the GPA1' was inserted into the expression vector inframe with *myc*-epitope as a C-terminal fusion. The expression control protein β-galactosidase is also fused with *myc*-epitope and it serves as control for the antibody binding as well as expression. Figure 4.21 shows the results; the anti-*myc* binding to 119 kDa β-galactosidase is clearly observed. For both controls lacZ and pPICZC it is seen that some unknown low molecular weight intracellulary expressed proteins also interact with the antibody. No signal corresponding to GPA1' could be detected on the same figure. Growth data relevant to induction is given in Appendix H.2.

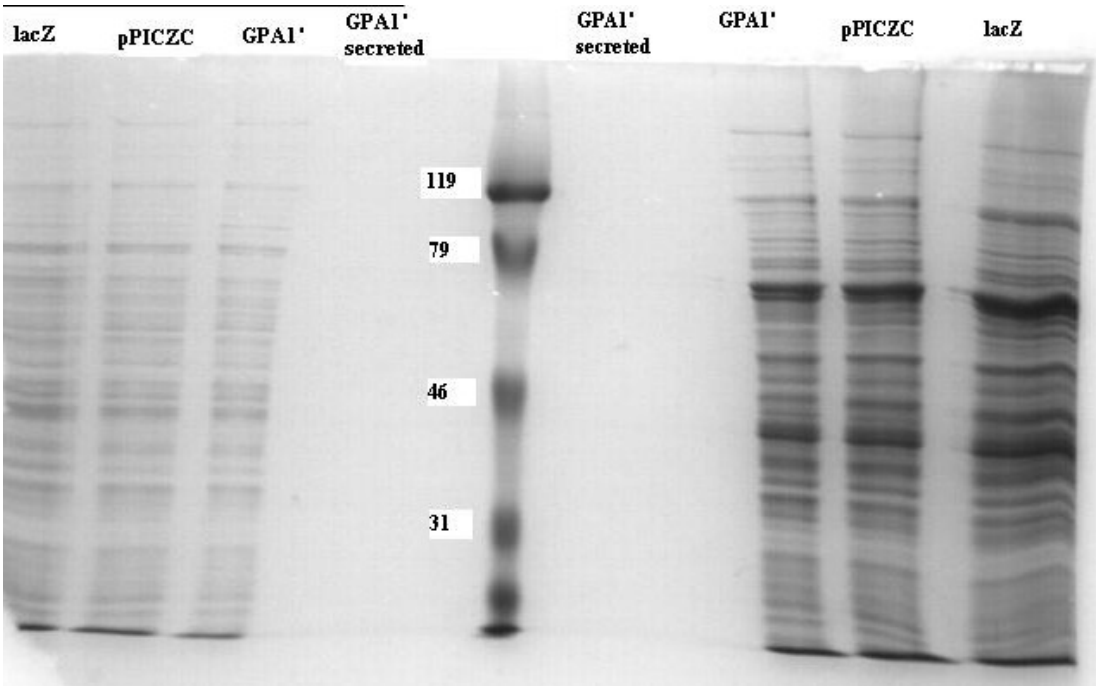


Figure 4.19 SDS-PAGE analysis of induction of control and GPA1' samples. Molecular weight marker proteins are loaded in the middle lane, samples taken after 6 hrs (left of the marker) and after 36 hours (right of the marker)

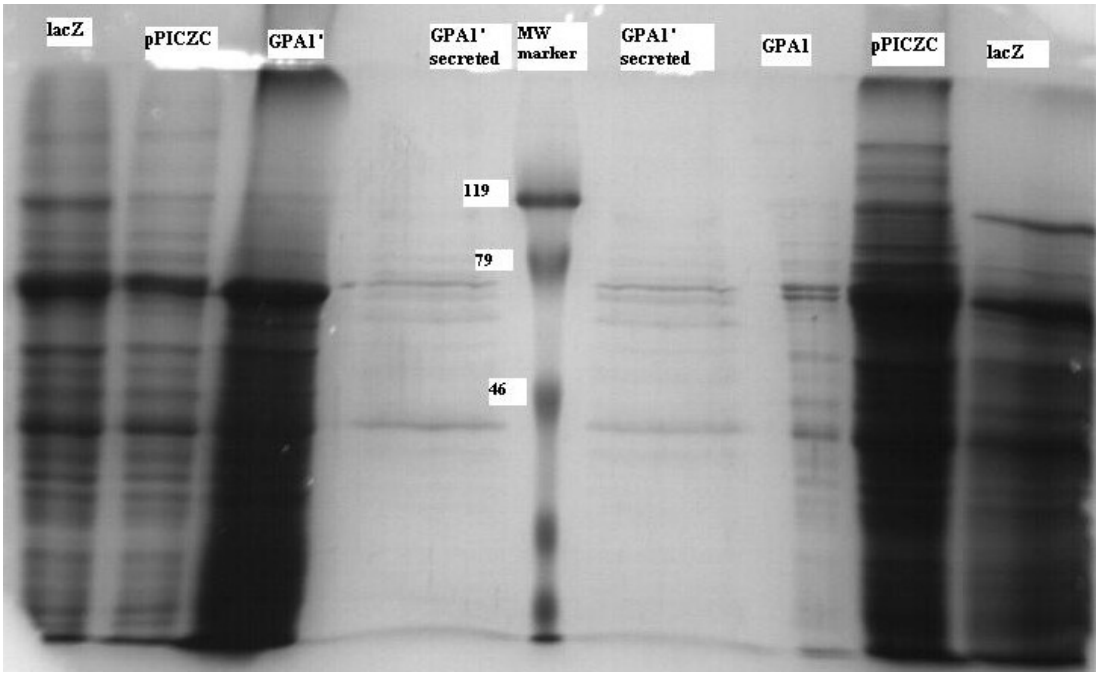


Figure 4.20 SDS-PAGE analysis of induction of control and GPA1' samples. Molecular weight marker proteins are loaded in the middle lane , samples taken after 60 hrs (left of the marker) and after 72 hours (right of the marker)

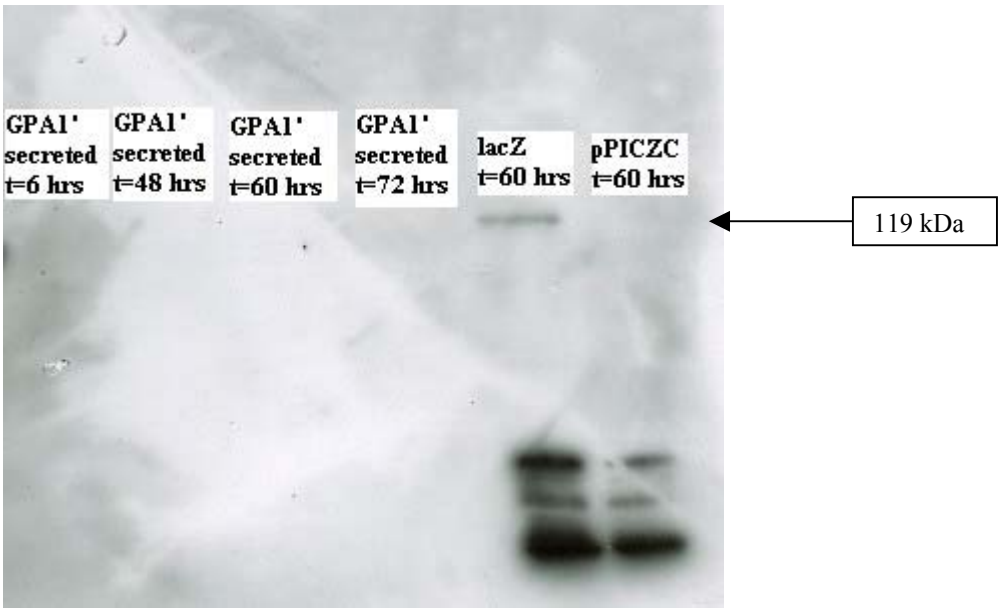


Figure 4.21 Result of Western Blot analysis of samples taken at different times during GPA1' induction.

4.5.2.2 Induction of GPA1 expression

Induction of *GPA1* encoding strain was analyzed on 10% SDS-PAGE and by Western blot using anti-*myc* antibody. An example of SDS-PAGE analysis is given in figure 4.22. Due to expression of large amounts of native proteins of *P. pastoris,* also at molecular weights corresponding to the size of β-galactosidase (119 kDa) and Gα (46 kDa), expression of these recombinant proteins could not be discerned directly by Coommassie blue staining. To overcome this problem gels of cell lysates were incubated with anti-*myc* for western blot analysis. As can be seen in figure 4.23 except for the sample taken after 6 hours of induction, all samples of *GPA1* encoding cell lysates contain a 46 kDa sized protein which specifically interacts with the antibody. Chemiluminescence seen in the sample taken from the control strain 10e (lane labeled pPICZC), indicates that the lower bands seen in all samples are likely to reflect antibody interaction with some background proteins. These results verify that the strain induced, GS115 1-1, expresses recombinant *A. thaliana* Gα and expression of the recombinant protein is continued even after 90 hours. The growth curves of induced cells are given in figure 4.24. After carbon source is switched to methanol, yeast cells enter the lag phase and as seen from figure this period lasts ~40 hours for expressing strains (A and

B) and ~20 hours for control strain 10e (C). OD_{600} measurements of GS115 integrants and GS115/ pPICZC/lacZ during induction; at the time points when samples for SDS-PAGE and Western Blot analyses were taken are given in table 4.5.

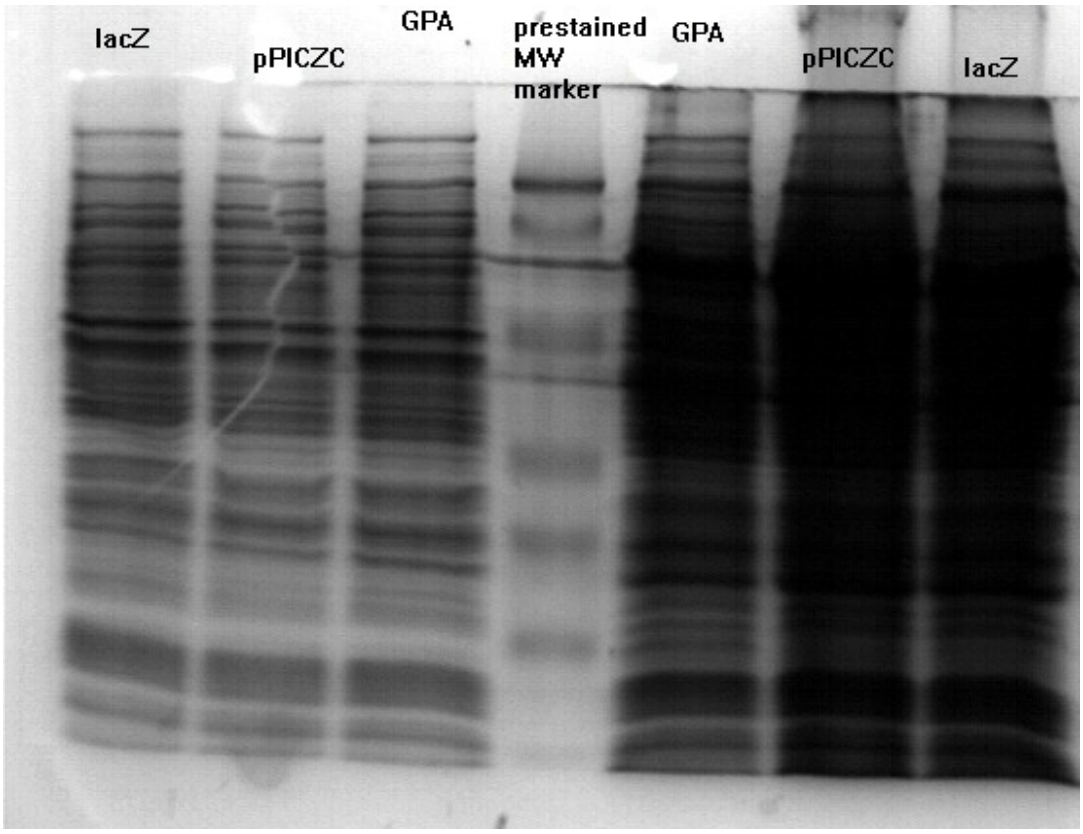


Figure 4.22 SDS-PAGE analysis of induction of control and GPA1 samples. Molecular weight marker proteins are loaded in the middle lane , samples taken after 6 hrs (left of the marker) and after 48 hours (right of the marker)

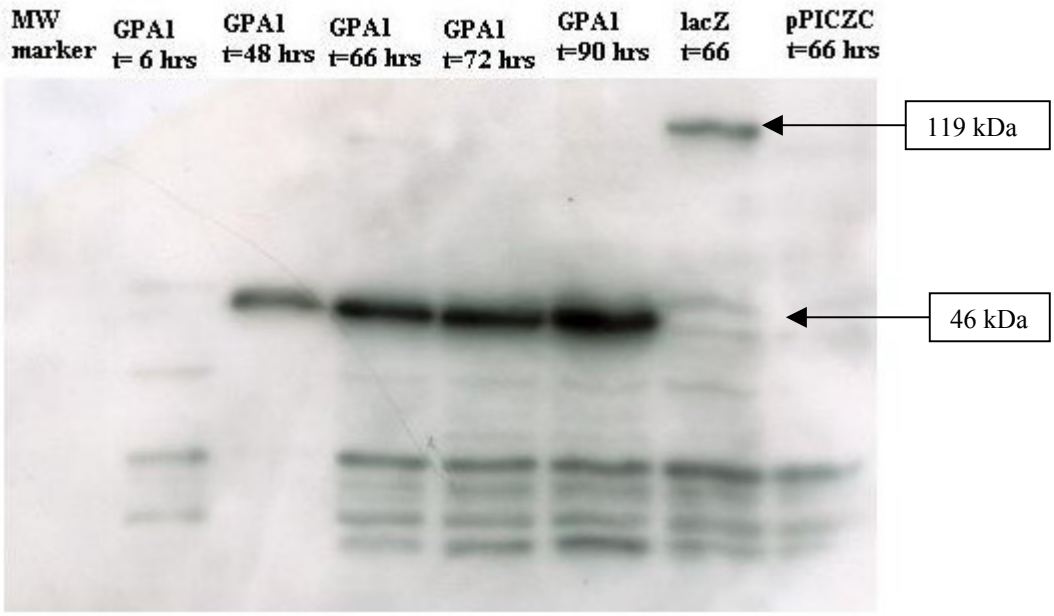
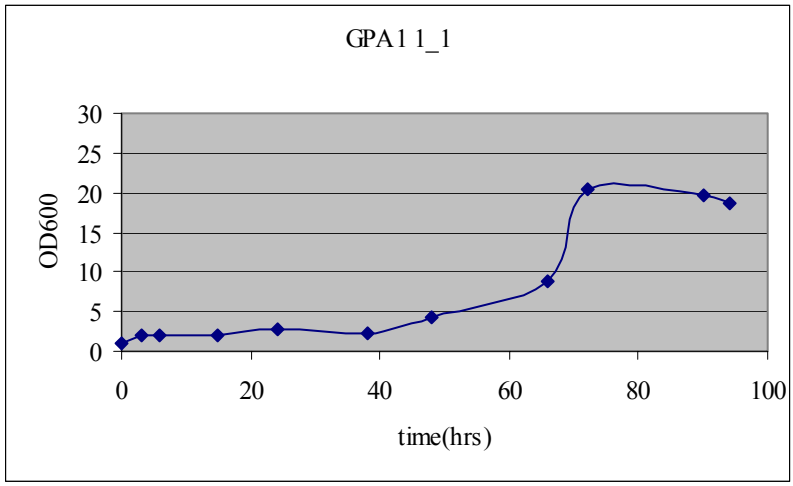
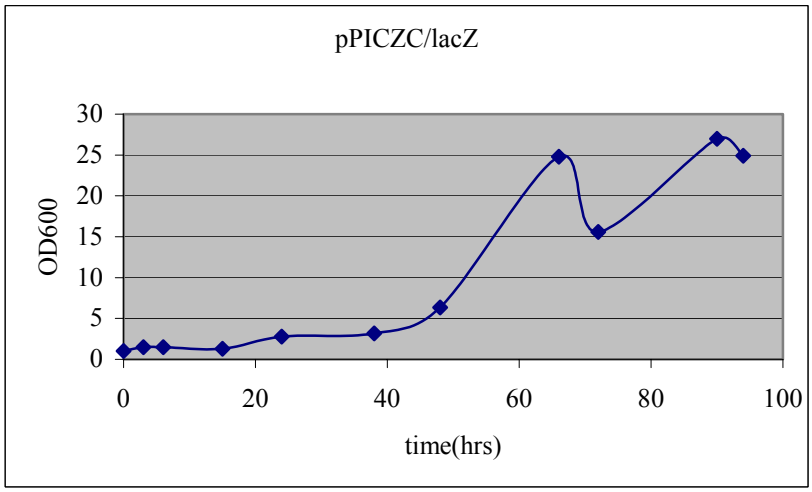


Figure 4.23 Detection of antibody binding after Western blotting, time lapsed after induction and the gene induced is indicated for each sample.

A



B



C

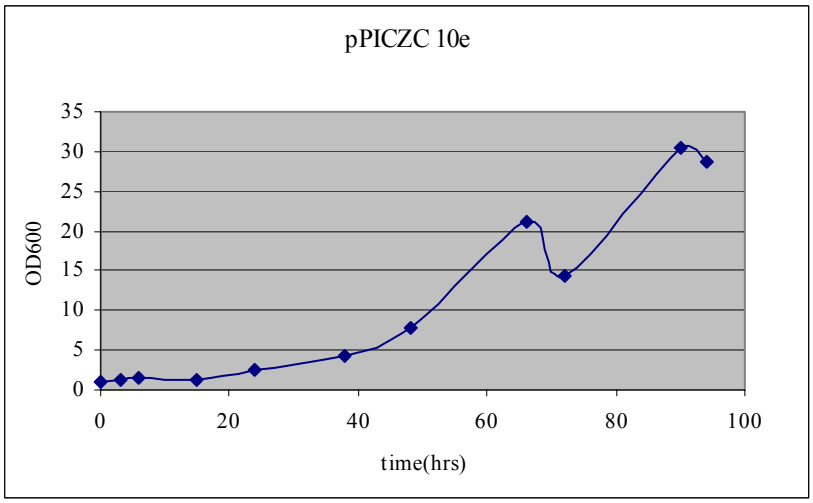


Figure 4.24 Growth curves of induced yeast cells, GS115 1-1(A), GS115/pPICZC/lacZ (B), GS115 10e (C).

Time (hours)	methanol	1_1	10e	pPIICZC/lacZ
0	+	1	1	1
3		2	1,3	1,5
6		2,06	1,5	1,5
15		2,12	1,2	1,3
24	+	2,8	2,6	2,8
38		2,18	4,22	3,18
48	+	4,4	7,8	6,35
66		8,8	21,25	24,8
72	+	20,3	14,4	15,6
90		19,75	30,5	27
94		18,65	28,6	24,9

Table 4.5 OD_{600} from cultures of GS115 integrants and GS115/ pPICZC/lacZ during induction.

5 DISCUSSION

The long-term aim of this study is to contribute to experimental determination of the structure of plant heterotrimeric G proteins with a view of understanding structure-function relationships. Although the 3D structures of *A. thaliana* G alpha subunit (Şahin, 2002) and the *A. thaliana* G protein heterotrimer have been modeled (Ullah *et al*.,2003) to enable functional predictions, direct structural information on the complex is lacking. Mutational and *in vivo* data suggest a role in signal transduction similar to mammalian heterotrimeric G proteins, but this prediction still awaits experimental proof. This study is the first report of cloning of the α subunit of the *A. thaliana* heterotrimer in a eukaryotic expression system with the aim of structural studies.

The mechanism of action of mammalian heterotrimeric G proteins, involving protein–protein interaction events, was elucidated after determination of crystal structures, in inactive and active conformations, of the α subunit, the βγ dimer and finally the heterotrimer. The importance of structural data in understanding the mechanism of GTP hydrolysis, the effect of nucleotide exchange on heterotrimer association/ dissociation and the mode of interactions of subunits with each other and upstream/ downstream molecules is reviewed by Bohm *et al*. (1997).The structural and complementary biochemical data, also help in identification of the functional domains and surface residues of subunits, and the possible agents that interact with these domains; receptors and effectors.

Structural studies require large amounts of highly purified proteins which can, in principle, be produced by use of molecular cloning and expression techniques. Several mammalian heterotrimeric G protein α subunits were expressed in both bacterial, *E. coli*, and baculovirus infected insect cells (Graziano *et al*., 1989, Graber *et al*., 1992). There are, however, limited reports in literature of studies of plant recombinant Gα

expression (Bakkal, 2003, Wise *et al*., 1997, Iwasaki *et al*., 1997, Seo *et al*., 1995 and Aharon *et al*., 1998). In one report *A. thaliana* Gα expression in *E. coli* (Wise *et al*,1997) was achieved by co-transformation of the host with the expression construct and *dnaY* gene which encodes $tRNA^{Arg}$, preventing premature termination of the translated protein. Unlike their mammalian counterparts plant Gα proteins are not easily expressed in *E. coli* without performing manipulations on the host (Bakkal, 2003). The work presented in this thesis describes our attempts to express *A. thaliana* Gα using a yeast expression system, *P. pastoris*. Two vectors, one giving rise to intracellular expression and the other facilitating secretion of the expressed protein into the extracellular medium, were tried using different yeast strains. The intracellular expression of recombinant Gα, rGpα1, over a relatively long time period was achieved. Expression was monitored by growth curves and protein analysis, and was confirmed by antibody labeling of the *myc*-epitope fused to rGpα1.

Basic features of strategies followed for cloning the *A. thaliana* Gα gene, *GPA1,* are illustrated in figure 5.1

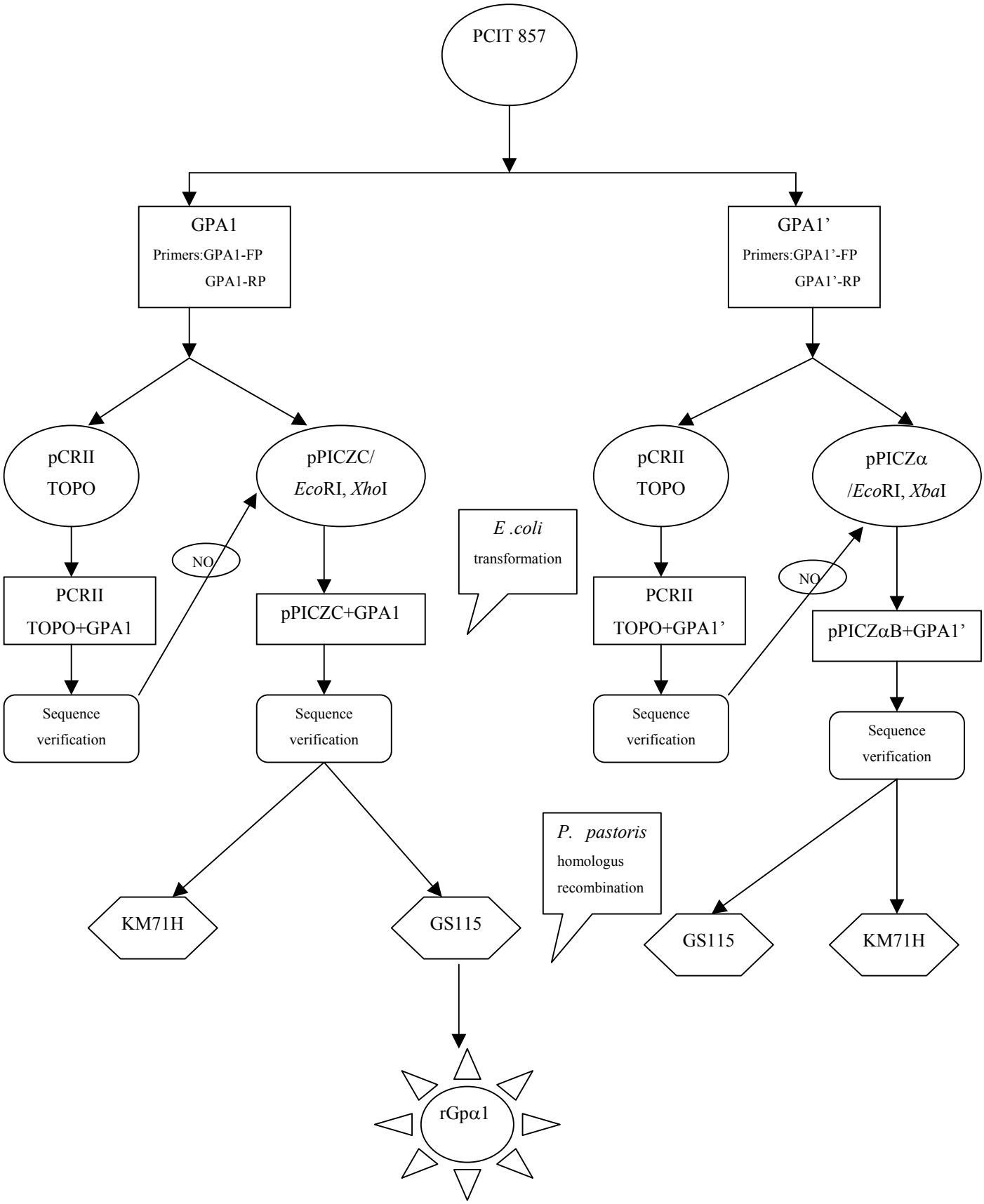


Figure 5.1Cloning strategies of *GPA1* using different vectors

5.1 Cloning

GPA1, was amplified from the plasmid PCIT 857, which was shown to contain the intact coding sequence (Ma *et al*., 1990, Bakkal, 2003). In order to facilitate in-frame insertion into two different yeast expression vectors two sets of primers, GPA1-FP & GPA1-RP and GPA1'-FP & GPA1'-RP, were designed, and the PCR product obtained using the former set was labeled *GPA1* and that of the latter set was labeled *GPA1*'. Amplification conditions had to be optimized for each set of primers by varying the duration of the annealing period of the PCR cycle since the length of the oligonucleotides were different (Figures 4.2 and 4.3).

The PCR product *GPA1* amplified with the forward primer, GPA1-FP was cloned using an intracellular yeast expression vector pPICZC. GPA1-FP contained a yeast consensus sequence which is required for the recognition of the promoter sequence by the transcriptional regulatory elements of yeast *P. pastoris. GPA1*'was amplified using, forward primer GPA1'-FP and was cloned using pPICZαB. The expression vector pPICZαB already contains the yeast consensus sequence within the *S. cerevisiae* secretion signal sequence, which leads to transport of recombinant proteins to extracellular medium. GPA1'-FP was designed to fuse *GPA1*' in frame with secretion signal. The *S. cerevisiae* α-factor prepro peptide consists of 19 amino acid signal (pre) sequence followed by a 66-residue (pro) sequence containing three consensus N-linked glycosylation sites and a dibasic Kex2 endopeptidase processing site (Kurjan and Herkowitz, 1982). The signal processing starts with the removal of the pre signal by a signal peptidase in the endoplasmic reticulum, followed by the cleavage of pro leader sequence between amino acids Arg and Lys by Kex2 endopeptidase and finally Glu-Ala repeats are cleaved by Ste13 protein (Brake *et al*.,1984). The close proximity proline residues can influence cleavage efficiencies of Kex2 and Ste3 proteins and the tertiary structure formed by a foreign protein may protect cleavage sites from these proteases.

Both expression vectors contain *myc*-epitope and his-tag sequences following the MCS. In order to fuse *GPA1* and *GPA1*' in frame with these sequences reverse primers of both genes, GPA1-RP and GPA1'-RP, were designed to exclude stop codons.

The first step, before integrating the constructs to the yeast genome, involved subcloning using the pCR II TOPO vector and the bacterial host *E. coli*. *E. coli* provided a well defined simple system for stable storage of the constructs as well as for isolation in large quantities for verification of the inserted gene sequences (Figure 4.4). It was envisaged that the inserts could be easily digested out of these constructs with the appropriate restriction enzymes for ligation with expression vectors.

Although *GPA1* and *GPA1*' were inserted into the subcloning vector and sequences were verified, digestion of neither *GPA1* nor *GPA1*' out of the constructs was efficient and enough material for subsequent steps could not be obtained. In order to optimize the reaction, several parameters including restriction enzyme concentration, reaction time and vector DNA concentration were changed. Even single digestion making use of the two flanking *Eco*RI sites of the subcloning vector, did not result in isolation of sufficient quantities of the insert. Failure of digestion may be attributed to 3D structural features that arise with the ligation of the insert. Since all optimization attempts were not successful (results not shown) alternative methods for cloning using expression vectors had to be explored.

Direct preparation of PCR products without the subcloning step appeared to be the next natural choice. Digestion of PCR products and expression vectors with the appropriate restriction enzymes and ligation of these components after purification by gel extraction was tried. *E. coli* TOP10F' cells were transformed with the ligation mixture but ligation efficiency was too low and it has not been possible to observe any colonies of transformed cells. Cloning was eventually achieved by the ethanol precipitation approach (Figures 4.9 and 4.11). Treatment of digested fragments together with vectors, the use of excess ethanol to remove unwanted components of previous enzymatic steps and forcing the ligation to take place in a very small volume increased the efficiency of the reaction. Sequencing results show that the inserts are correctly positioned in the constructs pPICZC+GPA1and pPICZαB+GPA1' (Appendix F).

Sequence verified constructs pPICZC+GPA1 and pPICZαB+GPA1' were linerized in order to integrate the foreign gene expression cassette into yeast genome via homologous recombination sites present at both upstream and downstream of the

foreign gene. *P. pastoris* strains GS115 and KM71H were most efficiently transformed by lithium chloride method where use of a carrier DNA and an alkali cation were provided the suitable conditions.

Integration of *GPA1* or *GPA1'* to the yeast genome could be verified by colony PCR performed for both strains using the 5'AOX and 3'AOX primers instead of gene-specific primers. As mentioned before (4.3.1) the use of gene specific primers may result in amplification of host genomic sequences and may give rise to false positives. *S. cerevisiae* encodes for a G protein α subunit, designated as *Gpa1p*, with 1419 nucleotides, corresponding to a protein composed of 472 amino acids (Sacchoromyces Genome Database). The nucleotide sequence of *Gpa1p* is 47.3% identical to that of *GPA1*. Although there are no reports it is highly likely that the genome of *P. pastoris* also contains a Gα gene, and this may be amplified by GPA1/GPA1' primers, interfering with PCR results.

GS115 transformants were also screened for Mut^+ phenotype, since integration events may disrupt the coding sequence of *AOX1* gene via recombination through the vectors' 3' AOX region. Several GS115 colonies were verified to utilize methanol at wild type levels, showing the *AOX1* gene is intact. The strain KM71H has already an insertion in AOX1 gene and thus all transformants are Mut^S.

5.2 Expression

Both the strains, GS115 and KM71H, are easily inducible by methanol at the promoter of *AOX1* gene. Although *AOX1* gene of the Mut^S strain KM71H is disrupted, the transcriptionally weaker *AOX2* gene is activated for methanol utilization and cells grow slower than Mut^+ strain GS115. Thus the expression conditions for the GS115 transformants and KM71H transformants were different.

KM71H transformants were grown in larger volumes of glycerol medium in order to obtain higher biomass before induction. On the other hand, GS115 transformants

were induced in larger volumes, since they showed better growth on methanol than KM71H cells. Final methanol concentration was kept constant and at 0.5% for both the strains.

5.2.1 Induction of KM71H transformants

The Mut^s strain GS115 Albumin was included as a positive control for expression and verified that Mut^s strains are induced by methanol. GS115 Albumin expresses extracellulary a 67 kDa protein and is seen on SDS-polyacrylamide gel (Figure 4.18). It was not possible to conclude on rGpα1 expression by clone 3 (Table 4.2) since control strains also express proteins with MW about 46 kDa intracellulary and our gels were not of high quality.

Induction data and expression results are not sufficient to derive conclusions from KM71H transformants' expression profile. It was not possible to further pursue induction studies using this strain due to time constraints. In further studies expression should be optimized and analyzed by more specific detection methods. This strain offers to be a good potential host for GPA1' expression because of slower growth on methanol and relatively low induction of expression. Cells may efficiently process the secretion signal sequence as reported by previous studies (Tschopp *et al*., 1987b, Cregg *et al*., 1987).

5.2.2 Extracellular expression of rGpα1 by GS115 transformants

The transformant 7e-2 was induced for extracellular expression of rGpα1. The expression control was GS115/pPICZC/*lac*Z, which intracellularly expresses a 119 kDa protein β-galactosidase. The initial SDS-PAGE results showed that especially high MW proteins are not separated efficiently on 12% polyacrylamide gels and 10% gels were used after this observation. The expression of β-galactosidase was detected on 10% polyacrylamide gels, after 36 hours of induction (Figure 4.19), but no expression and/or

secretion of rGpα1 was observed for 7e-2. After 60 hours of induction, 7e-2 secretes some proteins to the extracellular medium, but due to low concentration and large number of different proteins seen on gel, it was not possible to make any conclusions. To overcome this problem protein was detected using western blots-,but none of the 7e-2 samples taken at different time points did gave chemiluminescent signal (Figure 4.21). The efficient binding of antibody to the *myc*-epitope was verified by the chemiluminescence detected for *myc*-epitope fused β-galactosidase. The results also show that some intracellular proteins of both 9' (table 4.2) and GS115/pPICZC/*lac*Z react with anti-*myc*.

The fact that Gα is a membrane bound protein in the native host *A. thaliana* may explain this result. It is likely that recombinant Gα may undergo posttranslational modifications and interact with the yeast membrane. The *S. cerevisiae* secretion signal sequence works efficiently in *P. pastoris,* but mainly for recombinant proteins that are natively secreted. Posttranslational modifications are also important in determining the efficiency of secretion of a protein, since the overall fold will affect the processing of the signal sequence. Although *P. pastoris* is a better eukaryotic expression system than *S. cerevisiae* when posttranslational modifications are considered, it is still possible that the plant protein may have been modificated improperly. Finally the high amount of proteases secreted by *P. pastoris* to extracellular medium rapidly degrades recombinant proteins, this may also be a reason for the negative result of rGpα1 expression.

5.2.3 Intracellular expression of rGpα1 by GS115 transformants

The transformant 1-1 was induced for intracellular expression of rGpα1. Initial screening of expression was performed by 10% SDS-PAGE, and due to the high level of the background, also observed in the control strain 10e (Table 4.2), it had not been possible to discern expression of rGpα1 (Figure 4.22). In order to specifically detect the proteins of interest, the SDS-polyacrylamide gel was incubated with anti-*myc*. Through antibody binding rGpα1was detected in all samples induced more than 48 hours with methanol. The 46 kDa protein was not present in any of the controls. The

chemiluminescence signal observed with proteins corresponding to lower MWs were also observed for soluble fractions of the controls namely strain 10e and GS115/pPICZC/*lac*Z, verifying that they are components from the native proteins of transformed yeast (Figure 4.23).

Combination of data from growth curves and analysis of cell lysates indicate that the rGpα1 expression starts after ~40 hours of methanol feeding. The induced yeast cells remain in the lag phase during this period and then they enter the exponential phase of growth (Figure 4.24 (A)). The undetectable levels of expression corresponds to the lag phase of yeast cells, when they are trying to adopt the new carbon source. Similarly the strain expressing β-galactosidase remains in lag phase for 40 hours (Figure 4.24 (B)). Whereas 10e, which was obtained by integrating the unmodified parent vector pPICZC into GS115 genome, is growing much more faster than the expressing strains and is already in exponential phase after 40 hours of induction (Figure 4.24 (C)). This better growth of 10e can be explained by the absence of the metabolic burden of expressing and processing a foreign protein.

Western blot results given in this study (figure 4.23) can only be qualitatively interpreted due to inequalities in the amount of material loaded on gels and saturation of proteins with antibody. Lanes loaded with samples of cells induced more than 66 hours, are saturated therefore it is not possible to correlate the intensity of signals to protein concentration. The saturated lanes correspond to~$10*10^8$ cells/ml, whereas the lane without chemiluminsence at 46 kDa was taken from a culture with cell number of ~$10*10^7$/ml. Lanes for which lower MW proteins are detected, including lacZ and pPICZC are loaded with cell lysates corresponding to~$10*10^8$ cell/ml, 10 fold higher than those of giving very weak signals at these MWs. (Table 4.5).

A critical observation is that rGpα1 is not toxic for *P. pastoris*. The growth pattern of control strain GS115/pPICZC/*lac*Z expressing β-galactosidase, is similar to that of *GPA1* expressing strain 1-1. The toxicity of rGpα1 to *E. coli* cells was reported with the observation of no growth in expressing host cultures, even at the first hours of induction and immature termination of the recombinant protein due to its toxicity was discussed (Bakkal, 2003). The stability in the migration position of rGpa1 on

SDS_PAGE detected by western blots indicate that rGpα1 is neither degraded nor truncated by its host. However, although the cDNA sequence of *GPA1* is verified (Appendix F.3), the amino acid sequence of the recombinant protein should be controlled.

Whether rGpα1 retains its function or not is another point that needs clarification. This should be analyzed by GTPase activity and [^{35}S]GTP$_{\gamma}$S binding assays. Previous studies show that the recombinant *A. thaliana* enzymes retain their function when expressed in *P. pastoris* (Brandes *et al*., 1996, Bellevik *et al*., 2002 and Su *et al*., 1997). As mentioned before *P. pastoris* probably express its own G protein α subunit. The yeast Gα will not interfere with the activity assays, since rGpα1 will be purified to homogeneity via its fusion partner his-tag.

After verification of activity, the purified recombinant protein will be prepared for structural analysis. X-ray crystallography will provide static information at high resolution which will be useful in determination of the functional domains and facilitate comparison with mammalian counterparts. The conformational change upon nucleotide exchange can be monitored by X-ray small angle scattering and NMR. NMR is especially attractive since ^{13}C labeling of the recombinant protein can be achieved by feeding the expressing strains with ^{13}C-labelled methanol as the only carbon source. *P. pastoris* grows on ^{13}C-labelled methanol at wildtype levels and efficiently expresses the foreign protein (Laroche *et al*., 1994).

Finally the expression of *A. thaliana* Gα in *P. pastoris* provides evidence that the other subunits Gβ and Gγ may be expressed using this sytem. More importantly the *A. thaliana* heterotrimer can be expressed by *P. pastoris* by either co-expressing the three subunits in a haploid (Vuorela *et al*., 1997) or performing mating assays between Gα and Gβγ expressing haploids.

6 CONCLUSION

This study is the first report of cloning the *A. thaliana* Gα coding sequence using a yeast expression vector. The *P. pastoris* expression system resulted in the expression of rGpα1with the observations summarized below.

The sequence verified construct could be integrated properly into *P. pastoris* genome. The recombinant protein expression in Mut^+ *P. pastoris* strain can be induced by methanol. rGpα1 expressing strain GS115 shows a similar growth pattern to the control strain expressing β-galactosidase, revealing that expression does not negatively effect yeast growth. rGpα1 expression can be detected with antibody staining and possibly with coomasie blue staining if SDS-polyacrylamide gel electrophoresis conditions are optimized.

Future studies will concentrate on the biochemical and structural characterization of rGpα1. This studies are expected to contribute to current understanding of plant heterotrimeric G proteins by themselves and by comparison with well defined mammalian counterparts and modeled structures.

P. pastoris system used in this study provides a convenient expression system for plant proteins, including *A. thaliana* G protein β and γ subunits.

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APPENDIX A

CHEMICALS

(in alphabetical order)

Name of Chemical	Supplier Company	Catalog Number
Acetic Acid	Riedel-de Haén, Germany	27225
Acrylamide/Bis-acrylamide	Sigma, Germany	A3699
	Biorad Inc., USA	161-0158
Agar-agar	Merck, Germany	101614
(granulated)		
Agarose low EO	Applichem, Germany	A2114
Ammonium persulfate	Carlo-Erba, Italy	420627
Ampicillin	Sigma, Germany	A9518
Biotin	CALBIOCHEM, Germany	2031
β-galactosidase	Sigma, Germany	G-2531

Chloroform	Merck, Germany	102431
Coomassie Brilliant Blue	Merck, Germany	115444
Distilled water, sterile, MilliQ filtered	Millipore, France	
EDTA (Ethylenediamine tetraacetic acid)	Riedel-de Haén, Germany	27248
Ethanol	Riedel-de Haén, Germany	32221
Ethidium Bromide	Merck, Germany	OCO28942
D-(+) Glucose	Sigma, Germany	G-7021
Glycerol	Riedel-de Haén, Germany	15523
Glycine	Amnesa^{R}, USA	0167
HCl	Merck, Germany	100314
L-Histidine	Applichem, Germany	A3719
IPTG	Promega, Germany	V39517
Isopropanol	Riedel-de Haén, Germany	24137
Kanamycin	Sigma, Germany	K4000.102

KCl	Fluka, Switzerland	60129
KH_2PO_4	Riedel-de Haén, Germany	04243
KOH	Riedel-de Haén, Germany	06005
Liquid nitrogen	Karbogaz, Turkey	
Lithium chloride (anhydrous)	Fluka, Switzerland	62478
Luria Agar (Miller's LB Agar)	Sigma,Germany	L-3147
Luria Broth (Lennox L Broth)	Sigma, Germany	L-3022
2-Mercaptoethanol	Aldrich Chemical Company, Germany	M370-1
Methanol	Riedel-de Haén, Germany	24229
NaCl	Riedel-de Haén, Germany	13423
$NaO_2C_2H_3.3H_2O$	Riedel-de Haén, Germany	25022
NaOH	Merck, Germany	106462

$NaPO_4H_2$	Riedel-de Haén, Germany	04269
Peptone	Merck,Germany	107213
(from casein)		
PEG 6’000	Fluka, Switzerland	81253
(polyethylene glycol)		
Phenol	Applichem, Germany	A1153
Phenol/chloroform	Applichem, Germany	A0889
/isoamylalkohol		
Sodium Dodecyl Sulphate	Sigma, Germany	L-4390
D(-) Sorbitol	Applichem, Germany	A2222
TEMED	Sigma, Germany	T-7029
Triton^R X-100	Applichem, Germany	A1388
Tris	Fluka, Switzerland	93349
Tween® 20	Merck, Germany	822184
Yeast Extract	Applichem,Germany	A1552
Yeast Nitrogen Base	Invitrogen, Germany	Q300-07

(with ammonium sulfate without amino acids)		
Zeocin	Invitrogen, Germany	R250

APPENDIX B

MOLECULAR BIOLOGY KITS

(in alphabetical order)

Name of Kit	Supplier Company	Catalog Number
EasySelect ™ *Pichia* Expression Kit	Invitrogen, Germany	K1740-01
ECL Advance Western Blotting Detection Kit	Amersham Biosciences Sweden	RPN2135
Quiaquick® PCR Purification Kit (250)	Qiagen, Germany	28106
Quiaquick® Gel extraction Kit (250)	Qiagen, Germany	28706
Quiaprep® Spin Miniprep Kit (250)	Qiagen, Germany	27106
QIAGEN® Plasmid Midi Kit (100)	Qiagen, Germany	12145
QIAGEN® Plasmid Maxi Kit (500)	Qiagen, Germany	12165
TOPO® TA Cloning Kit	Invitrogen, Germany	K4600

APPENDIX C

OTHER MATERIALS

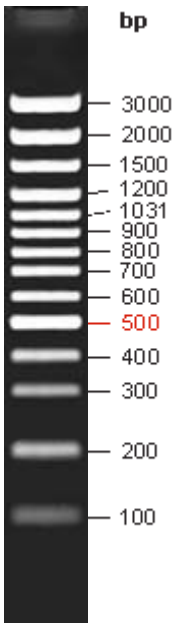
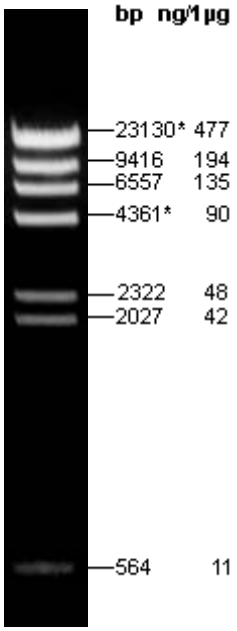
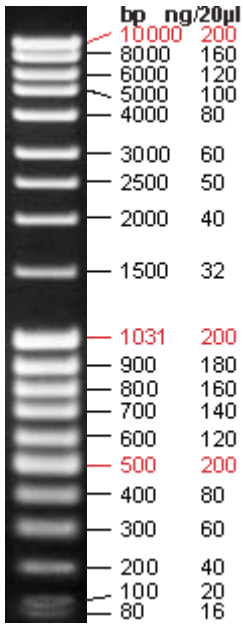
(in alphabetical order)

NAME OF ENZYME / BUFFER SYSTEM	Supplier Company	Catalog Number
100 bp DNA ladder+ (Agarose gel photograph and MW values of bands are provided below)	Fermentas, Germany	#SM0321
BstXI	Fermentas, Germany	#ER1021
Calf Intestine Alkaline Phosphatase	Fermentas, Germany	#EF0341
CYROBANK MIXED COLOURS	MAST GROUP Ltd, UK	Cyro/M
Deoxyribonucleic acid Sodium Salt from salmon testes	Sigma, Germany	9007-49-2
*Eco*RI	Fermentas, Germany	#ER0271

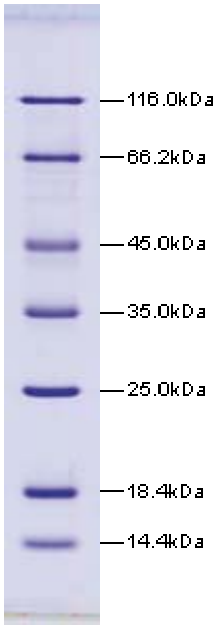
Glass beads 0.5 mm d	Biospec Products,Inc, US.	11079105
Hybond-P membrane (PVDF)	.Amersham Biosciences Sweden	RPN2020F
Hyperfilm ECL	Amersham Biosicences Sweden	RPN2103K
Lamda DNA/HindIII (Agarose gel photograph and MW values of bands are provided below)	Fermentas, Germany	#SM0123
Mass Ruler DNA Ladder,Mix (Agarose gel photograph and MW values of bands are provided below)	Fermentas, Germany	#SM0403
Myc tag antibody (HRP)	abcam, UK	ab1326
pCIT 857	was kindly donated by Dr.. Hong Ma (PennState University,USA)	
Pichia pastoris strains	Invitrogen, Germany	Supplied with EasySelect™ *Pichia* Expression Kit

pCR® II- TOPO®	Invitrogen, Germany	Supplied with TOPO® TA Cloning Kit
pPICZαB and PPICZC	Invitrogen, Germany	Supplied with EasySelect™ *Pichia* Expression Kit
Protein MW Marker (gel photograph and MW values of bands are provided below)	Fermentas, Germany	#SM0431
Prestained Protein MW Marker (gel photograph and MW values of bands are provided below)	Fermentas,Germany	#SM0441
RNAase	Qiagen, Germany	Supplied.with Qiagen Plasmid Isolation kits
*Sac*I	Fermentas, Germany	#ER1131
T4 DNA Ligase	Promega, Germany	M180B
T4 DNA Ligase Buffer (10X)	Promega, Germany	Supplied with T4 DNA Ligase

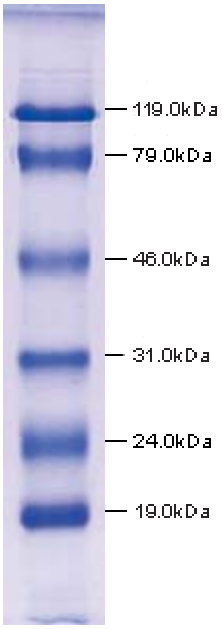
Taq DNA polymerase	Fermentas, Germany	#EP0401
TOP10	Invitrogen, Germany	Supplied with TOPO® TA Cloning Kit
TOP10F'	Invitrogen, Germany	Supplied with EasySelect™ *Pichia* Expression Kit
*Xba*I	Fermentas, Germany	#ER0681
*Xho*I	Fermentas, Germany	#ER0691
XL1-Blue	Kindly provided byEMBL, Hamburg Germany	

100 bp.DNA Ladder Plus	Lambda DNA/*Hin*dIII	Mass Ruler DNA LadderMix
		
1.7% agarose	1.0% agarose	1.0% agarose

Protein MW Marker



Prestained Protein Molecular Weight Marker



12% SDS-PAGE

Coomassie Brilliant Blue R-250 stained

APPENDIX D

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA
Balance:	Sartorius, BP211D, GERMANY
	Sartorius, BP221S, GERMANY
	Sartorius, BP610, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Blot Module	X Cell II ™ Blot Module, Novex, USA
Centrifuge:	Eppendorf, 5415C, GERMANY
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY
	Hitachi, Sorvall RC5C Plus, USA
	Hitachi, Sorvall Discovery 100 SE, USA

Deepfreeze:	-70^{o} C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY
	-20^{o} C, Bosch, TÜRKİYE
Distilled Water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
	X Cell SureLock ™ Electrophoresis Cell, Novex USA
Electroporation device	BTX^{R} ECM 630, A Division of Genetronics, Inc, USA
	BTX^{R} Safety Stand 630 B
Gel Documentation:	UVITEC, UVIdoc Gel Documentation System, UK
	Biorad, UV-Transilluminator 2000, USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
Laminar Flow:	Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY

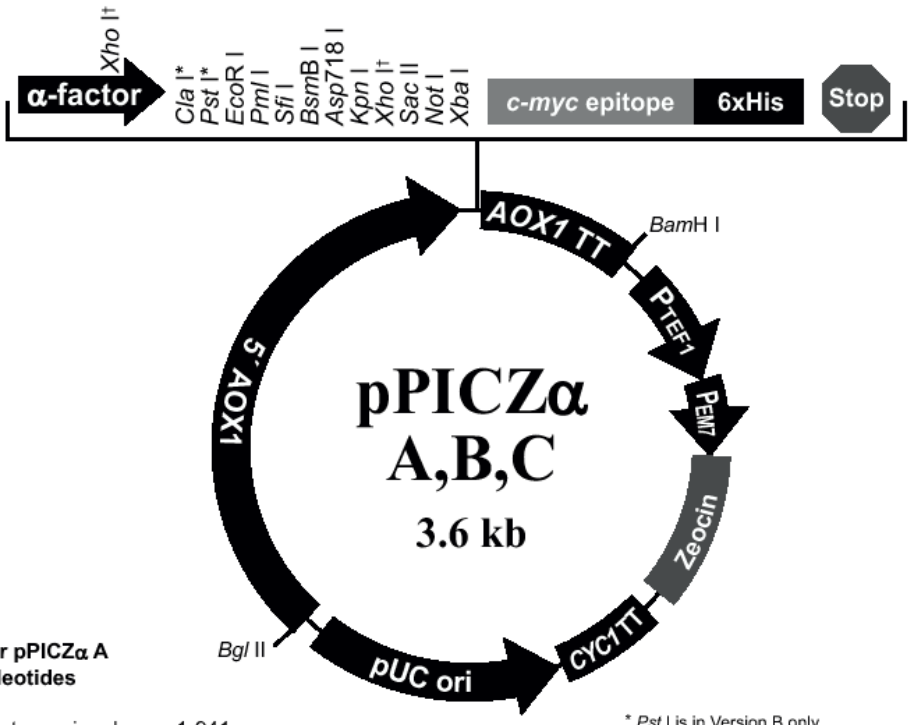
	VELP Scientifica, Microstirrer, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Mettler Toledo, Volumate, USA
Microwave Oven:	Bosch, TÜRKİYE
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
	Wealtec, Elite 300, USA
Refrigerator:	+4^{o} C, Bosch, TÜRKİYE
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., Innova™ 4330, USA
	C25HC Incubator shaker New Brunswick Scientific, USA
Sonicator	Vibracell 75043, Bioblock Scientific,FRANCE
Spectrophotometer:	Schimadzu, UV-1208, JAPAN
	Schimadzu, UV-3150, JAPAN
	Secoman, Anthelie Advanced, ITALY

Speed Vacuum:	Savant, Speed Vac® Plus Sc100A, USA
	Savant, Refrigerated Vapor Trap RVT 400, USA
Thermocycler:	Eppendorf, Mastercycler Gradient, GERMANY
Vacuum:	Heto, MasterJet Sue 300Q, DENMARK
Water bath:	Huber, Polystat cc1, GERMANY

APPENDIX E

VECTOR MAPS

E.1 Vector map of pPICZαB



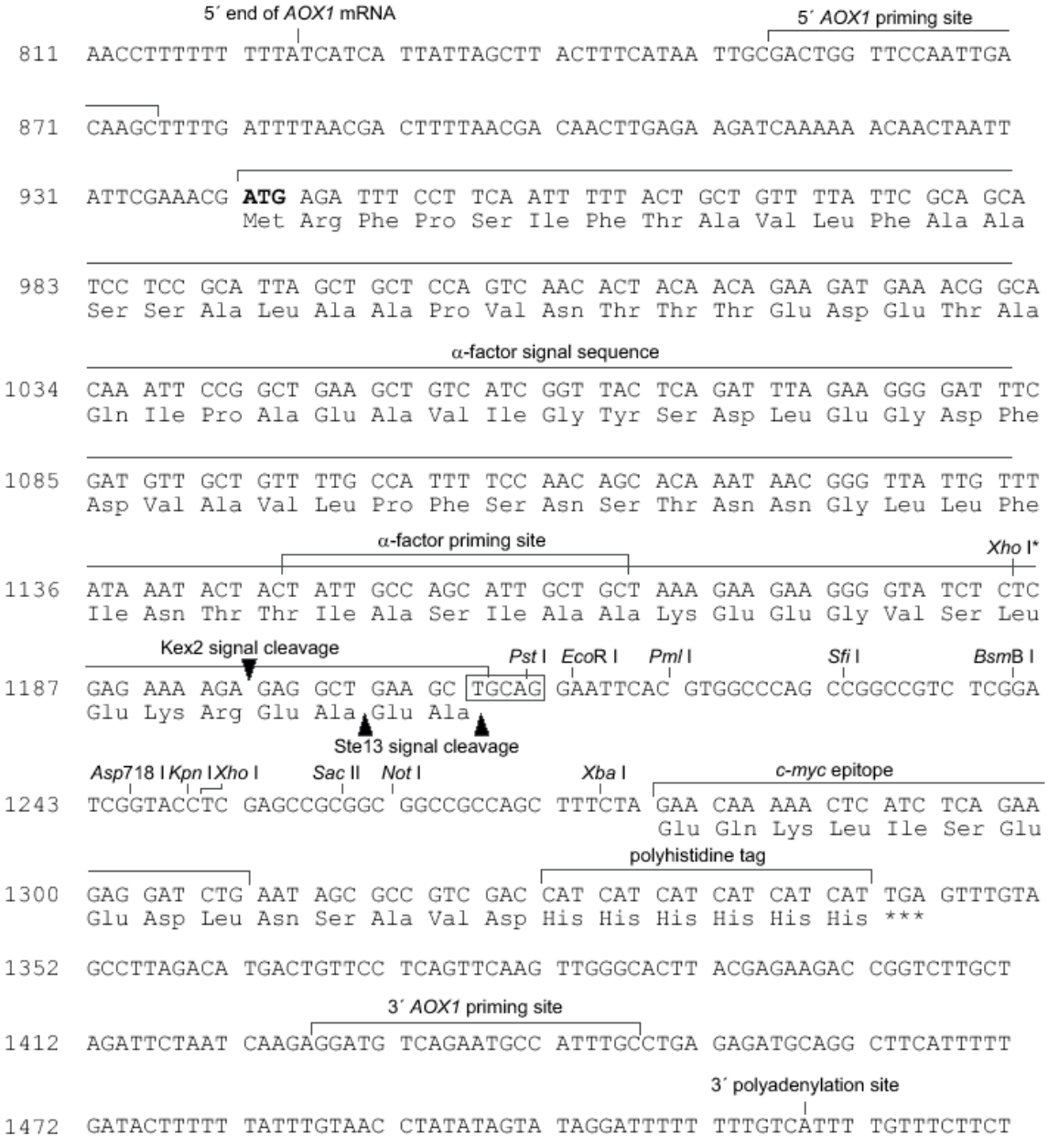
Comments for pPICZα A
3593 nucleotides

5´ *AOX1* promoter region: bases 1-941
5´ *AOX1* priming site: bases 855-875
α-factor signal sequence: bases 941-1207
α-factor priming site: bases 1144-1164
Multiple cloning site: bases 1208-1276
c-myc epitope: bases 1275-1304
Polyhistidine (6xHis) tag: bases 1320-1337
3´ *AOX1* priming site: bases 1423-1443
AOX1 transcription termination region: bases 1341-1682
TEF1 promoter: bases 1683-2093
EM7 promoter: bases 2095-2162
Sh ble ORF: bases 2163-2537
CYC1 transcription termination region: bases 2538-2855
pUC origin: bases 2866-3539 (complementary strand)

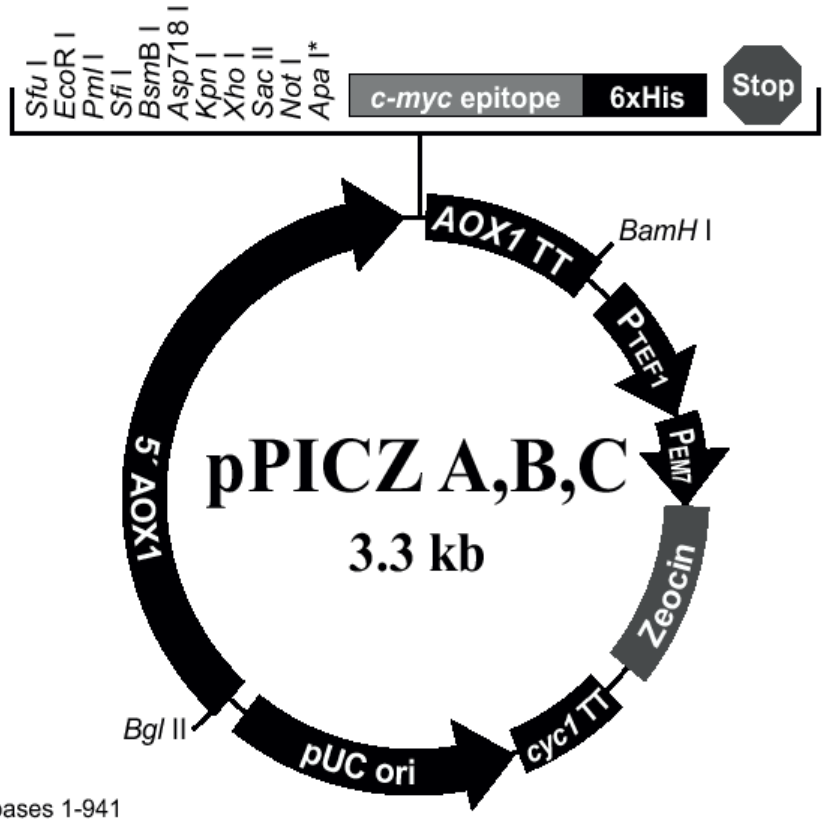
* *Pst* I is in Version B only
Cla I is in Version C only

†The two *Xho* I sites in the vector allow the user to clone their gene in frame with the Kex2 cleavage site, resulting in expression of their native gene without additional amino acids at the N-terminus.

Multiple cloning site of pPICZαB



E.2 Vector map of pPICZC



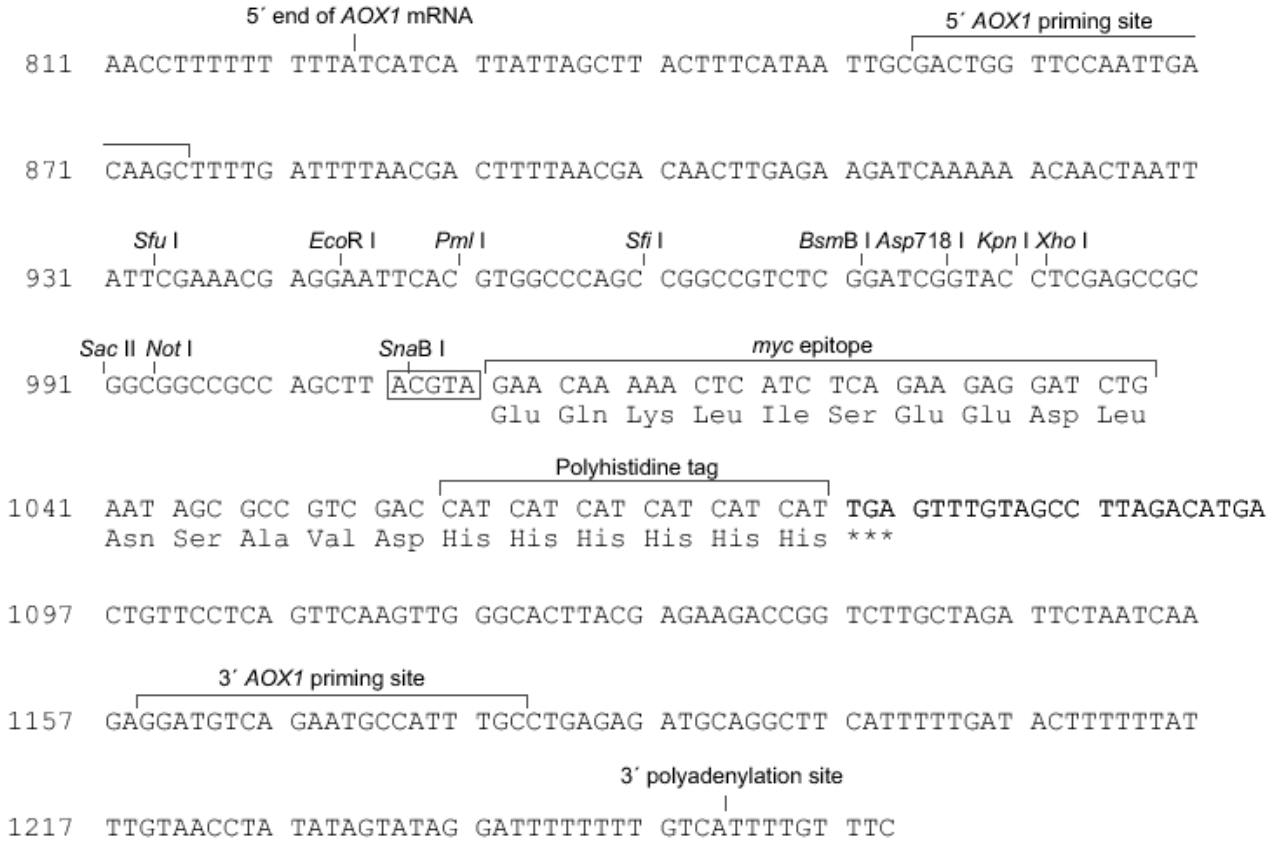
Comments for pPICZ A:
3329 nucleotides

5´ *AOX1* promoter region: bases 1-941
5´ end of *AOX1* mRNA: base 824
5´ *AOX1* priming site: bases 855-875
Multiple cloning site: bases 932-1011
c-myc epitope tag: bases 1012-1044
Polyhistidine tag: bases 1057-1077
3´ AOX priming site: bases 1159-1179
3´ end of mRNA: base 1250
AOX1 transcription termination region: bases 1078-1418
Fragment containing TEF1 promoter: bases 1419-1830
EM7 promoter: bases 1831-1898
Sh ble ORF: bases 1899-2273
CYC1 transcription termination region: bases 2274-2591
pUC origin: bases 2602-3275 (complementary strand)

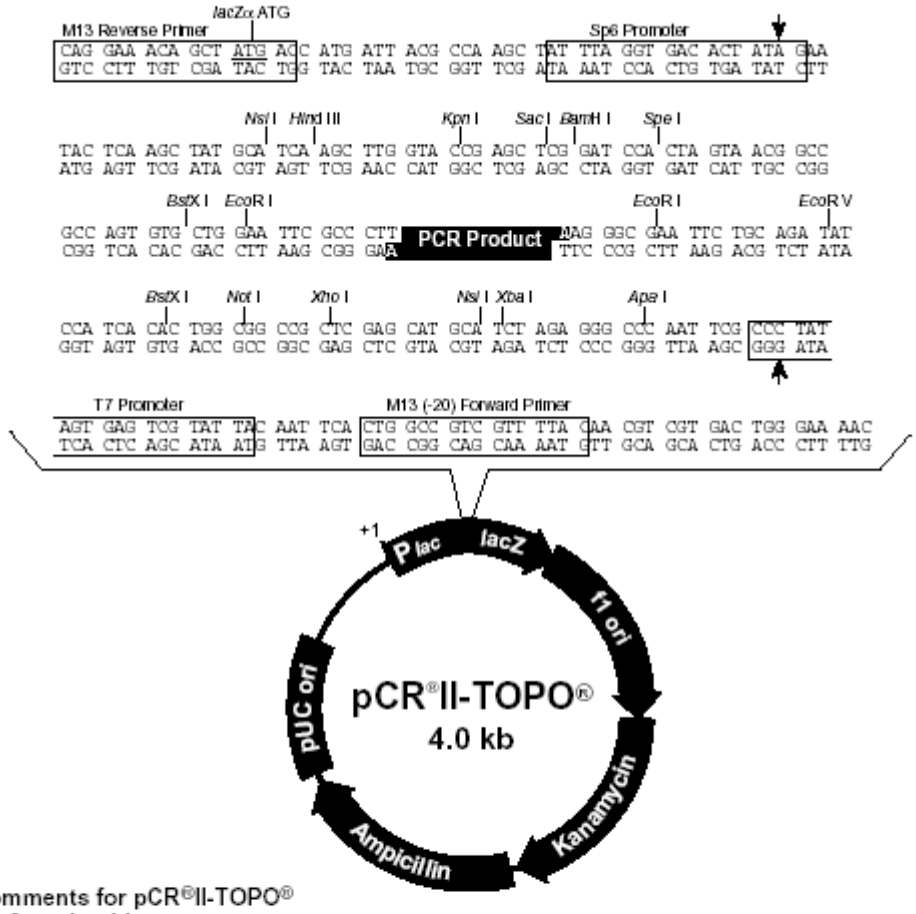
* The restriction site between *Not* I and the *myc* epitope is different in each version of pPICZ:

Apa I in pPICZ A
Xba I in pPICZ B
*Sna*B I in pPICZ C

Multiple cloning site of pPICZC



E.3 pCRII-TOPO vector map and sequence reference points



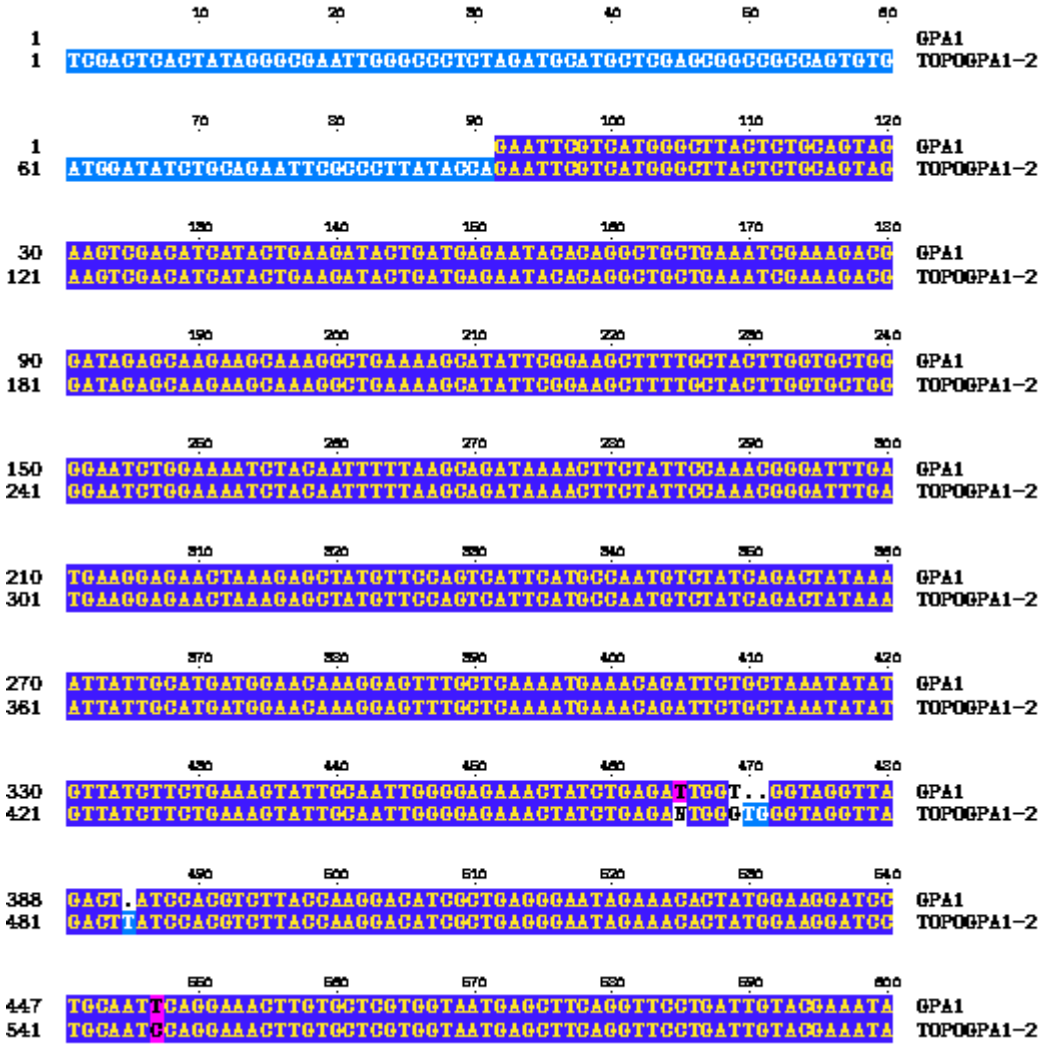
Comments for pCR®II-TOPO®
3973 nucleotides

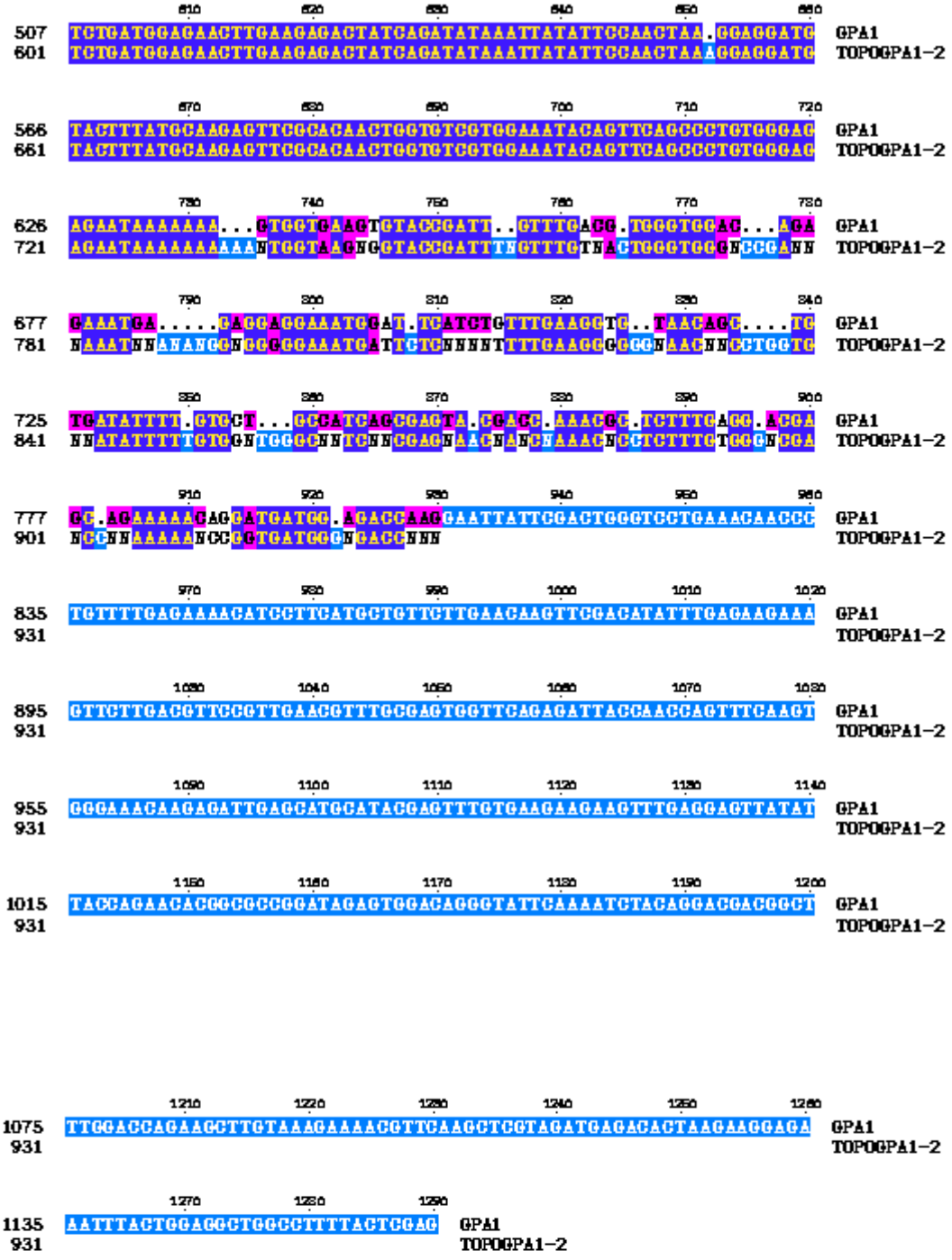
LacZα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

APPENDIX F

SEQUENCING RESULTS

F.1 Sequencing results of GPA1 and TOPO+GPA1-2 construct, with *Eco*RI and *Xho*I restriction enzyme sites at 5' and 3' sites, respectively

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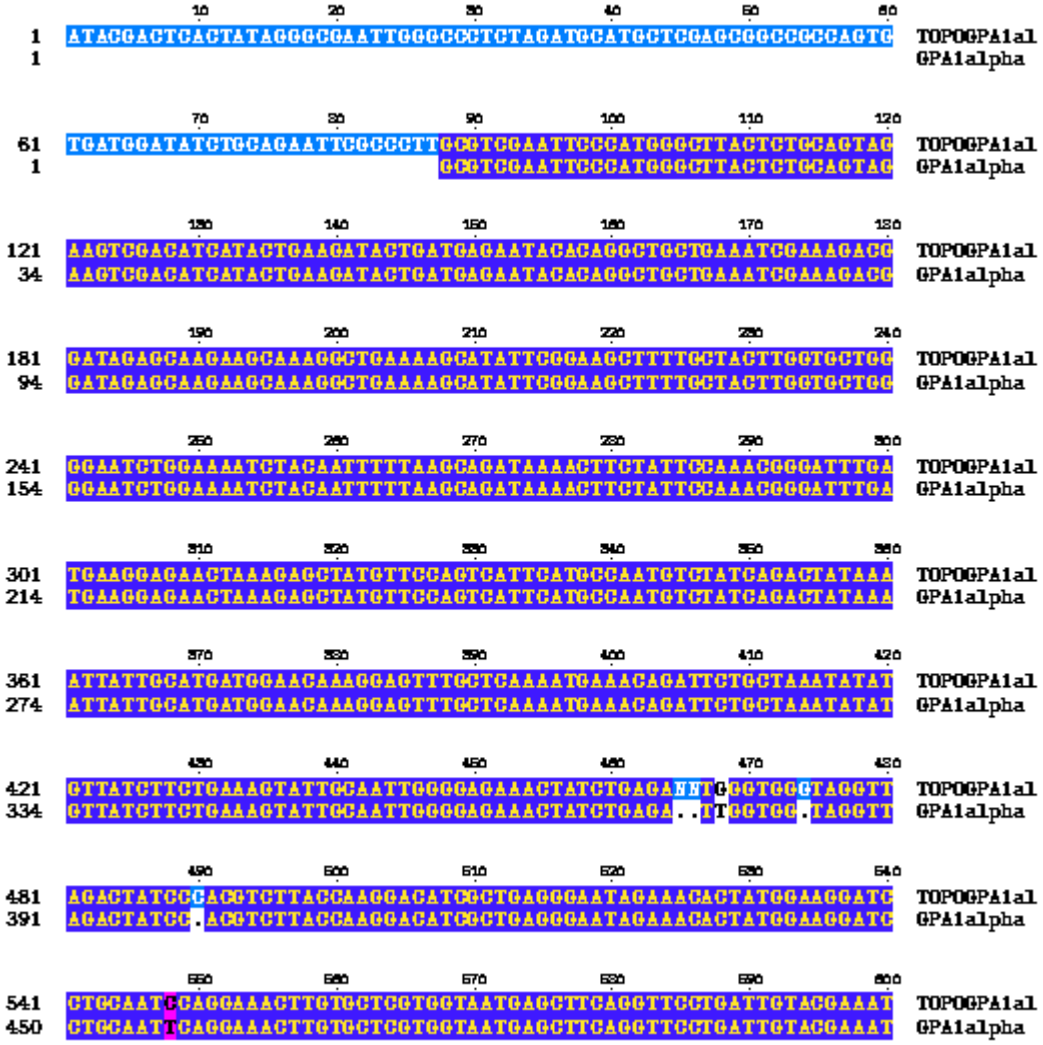
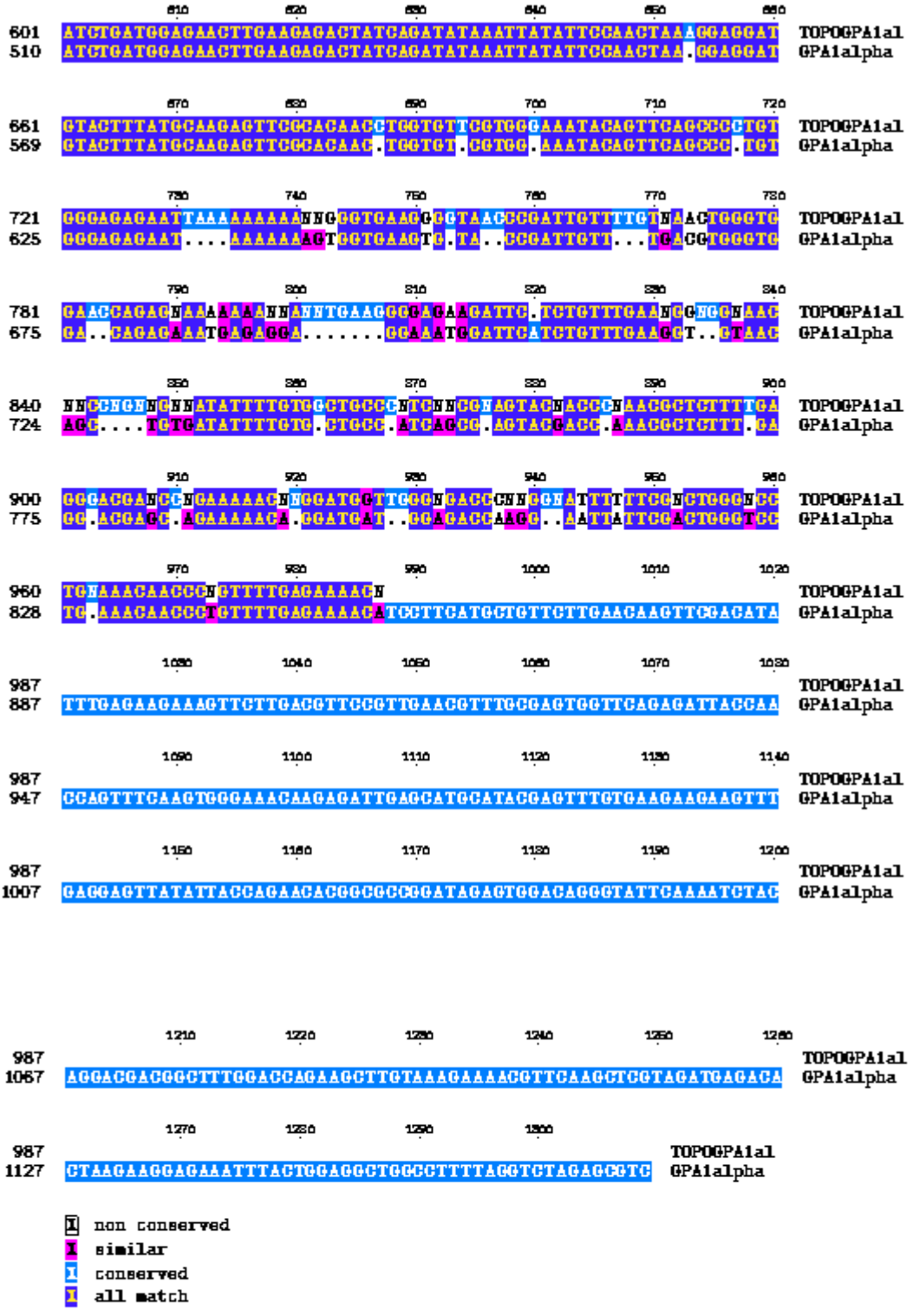
670 680 690 700 710 720
566 TACTTTATGCAAGAGTTCGCACAACTGGTGTCGTGGAAATACAGTTCAGCCCTGTGGGAG GPA1
661 TACTTTATGCAAGAGTTCGCACAACTGGTGTCGTGGAAATACAGTTCAGCCCTGTGGGAG TOPOGPA1-2
730 740 750 760 770 780
626 AGAATAAAAAAA...GTGGTGAAGTGTACCGATT..GTTTGACG.TGGGTGGAC...AGA GPA1
721 AGAATAAAAAAAAAAANTGGTAAGNGGTACCGATTTNGTTTGTNACTGGGTGGGNCCGANN TOPOGPA1-2
790 800 810 820 830 840
677 GAAATGA.....GAGGAGGAAATGGAT.TCATCTGTTTGAAGCTC..TAACAGC....TG GPA1
781 NAAATNNANANCGNGGGGGAAATGATTCTCNNNNTTTGAAGCGGGCNAACNNCCTGGTG TOPOGPA1-2
850 860 870 880 890 900
725 TGATATTTT.GTGCT...GCCATCAGCGACTA.CGAGC.AAACGC.TCTTTGAGG.ACGA GPA1
841 NNATATTTTTGTGGNTGGGCNNTCNNCGACNAACNANCNAAACNCCTCTTTGTGGGNCGA TOPOGPA1-2
910 920 930 940 950 960
777 GC.AGAAAAACAGGATGATGG.AGACGAAGGAATTATTCGACTGGGTCCTGAAACAACGC GPA1
901 NCCNNAAAAANCCGGTGATGGGNGACGNNN TOPOGPA1-2
970 980 990 1000 1010 1020
835 TGTTTTGAGAAAACATCCTTCATGCTGTTCTTGAACAAGTTCGACATATTTGAGAAGAAA GPA1
931 TOPOGPA1-2
1030 1040 1050 1060 1070 1080
895 GTTCTTGACGTTCCGTTGAACGTTTGCGAGTGGTTCAGAGATTACCAACCAGTTTCAAGT GPA1
931 TOPOGPA1-2
1090 1100 1110 1120 1130 1140
955 GGGAAACAAGAGATTGAGCATGCATACGAGTTTGTGAAGAAGAAGTTTGAGGAGTTATAT GPA1
931 TOPOGPA1-2
1150 1160 1170 1180 1190 1200
1015 TACCAGAACACGGCGCCGGATAGAGTGGACAGGGTATTCAAAATCTACAGGACGACGGCT GPA1
931 TOPOGPA1-2
1210 1220 1230 1240 1250 1260
1075 TTGGACCAGAAGCTTGTAAAGAAAACGTTCAAGCTCGTAGATGAGACACTAAGAAGGAGA GPA1
931 TOPOGPA1-2
1270 1280 1290
1135 AATTTACTGGACGCTGGCCTTTTACTCGAG GPA1
931 TOPOGPA1-2
non conserved
similar
conserved
all match

F.2 Sequencing results of *GPA1* and TOPO+GPA1'-3 construct, with *Eco*RI and *Xba*I restriction enzyme sites at 5' and 3' sites, respectively

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661 GTACTTTATGCAAGAGTTCGCACAACCTGGTGTTCGTGGGAAATACAGTTCAGCCCCTGT TOPOGPA1al
569 GTACTTTATGCAAGAGTTCGCACAAC.TGGTGT.CGTGG.AAATACAGTTCAGCCC.TGT GPA1alpha
721 GGGAGAGAATTAAAAAAAAANNGGGTGAAGCGGTAACCGGATTGTTTTGTNAACTGGGTG TOPOGPA1al
625 GGGAGAGAAT....AAAAAAAGTGGTGAAGTG.TA..CCGATTGTT...TGACGTGGGTG GPA1alpha
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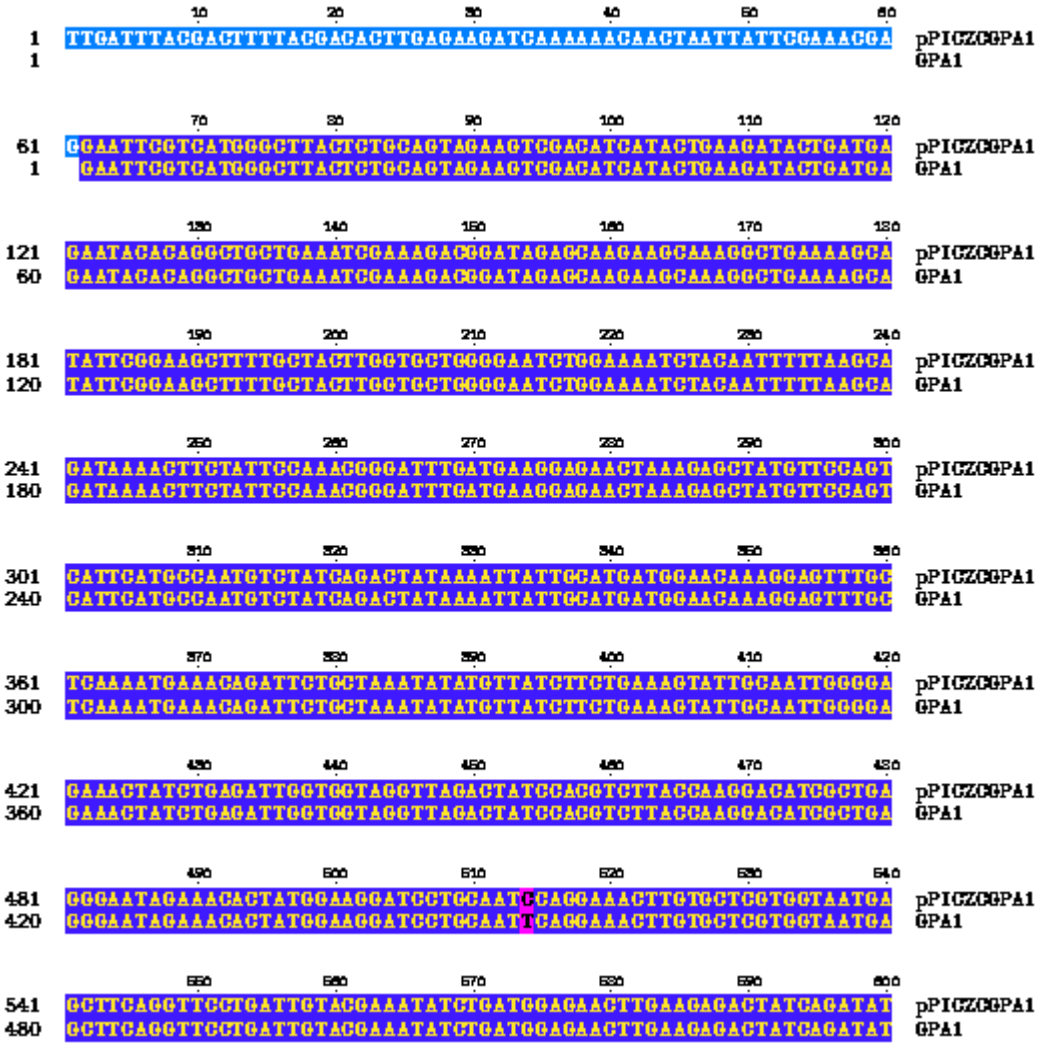
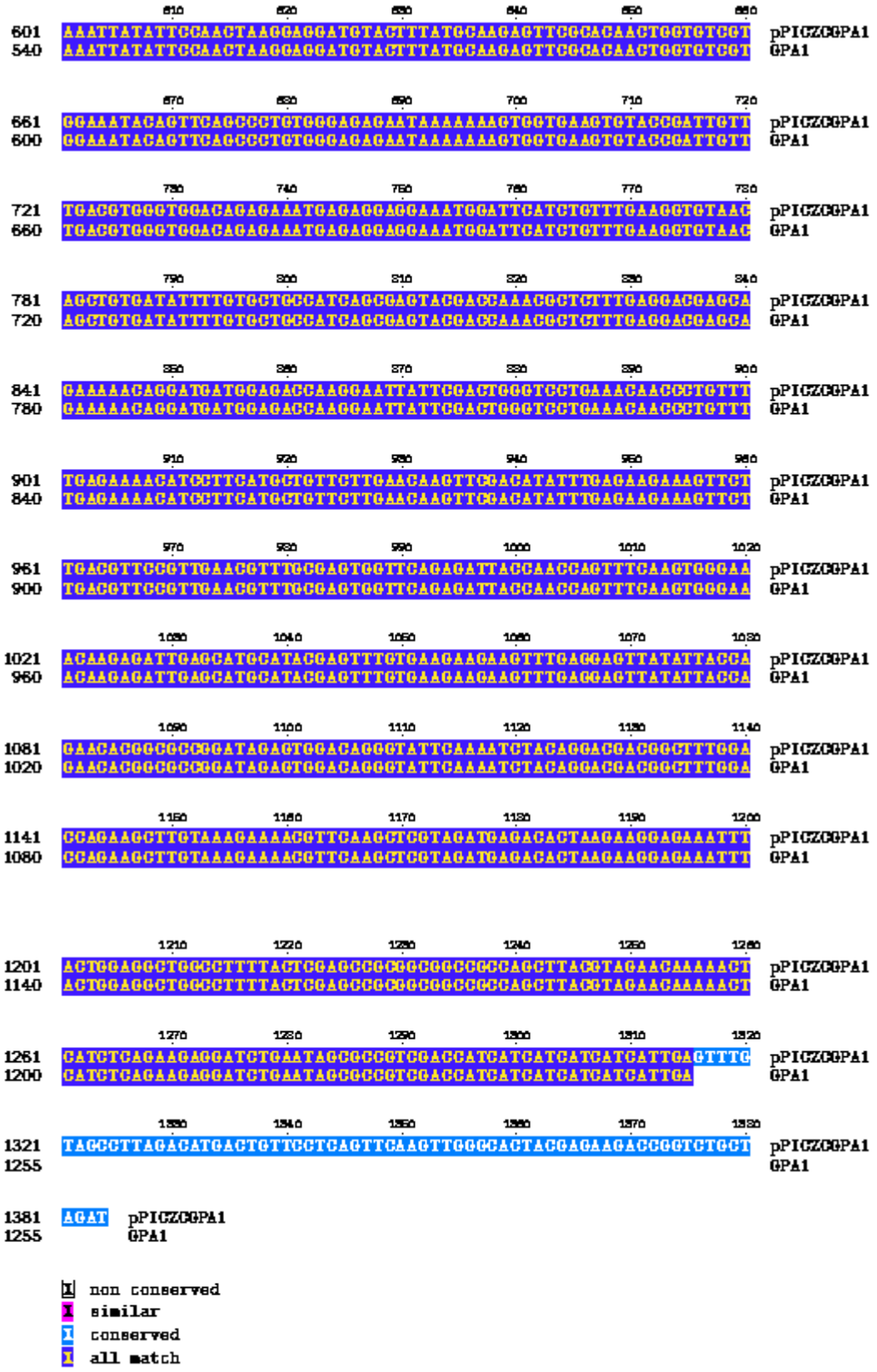
724 AGC....TGTGATATTTTGTG.CTGCC.ATCACGC.AGTACGACC.AAACGCTCTTT.GA GPA1alpha
900 GGCACGANCCNGAAAAACNNGGATCGTTCGCNGACCCNNCGNATTTTTCGNCTGGGNCC TOPOGPA1al
775 GG.ACGAGC.AGAAAAACA.GGATCAT..GGAGACCAAGG..AATTATTCGACTGGGTCC GPA1alpha
960 TGNAAACAACCGNGTTTTGAGAAAACN TOPOGPA1al
828 TG.AAACAACCGTGTTTTGAGAAAACATCCTTCATGCTGTTCTTGAACAAGTTCGACATA GPA1alpha
987 TOPOGPA1al
887 TTTGAGAAGAAAGTTCTTGACGTTCCGTTGAACGTTTGCGAGTGGTTCAGAGATTACCAA GPA1alpha
987 TOPOGPA1al
947 CCAGTTTCAAGTGGGAAACAAGAGATTGAGCATGCATACGAGTTTGTGAAGAAGAAGTTT GPA1alpha
987 TOPOGPA1al
1007 GAGGAGTTATATTACCAGAACACGGCGCCGGATAGAGTGGACAGGGTATTCAAAATCTAC GPA1alpha
987 TOPOGPA1al
1067 AGGACGACGGCTTTGGACCAGAAGCTTGTAAAGAAAACGTTCAAGCTCGTAGATGAGACA GPA1alpha
987 TOPOGPA1al
1127 CTAAGAAGGAGAAATTTACTGGAGGCTGGCCTTTTAGGTCTAGAGCGTC GPA1alpha
non conserved
similar
conserved
all match

F.3 Sequencing results of GPA1 and pPICZC+GPA1-2 construct, with *Eco*RI and *Xho*I restriction enzyme sites at 5' and 3' sites, respectively. Alignment starts at the EcoRI site and ends at the stop codon of polyhistidine tag of pPICZC.

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660 TGACGTGGGTGGACAGAGAAATGAGAGGAGGAAATGGATTCATCTGTTTGAAGGTGTAAC GPA1
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720 AGCTGTGATATTTTGTGCTGCCATCAGCGAGTACGACCAAACGCTCTTTGAGGACGAGCA GPA1
841 GAAAAACAGGATGATGGAGACCAAGGAATTATTCGACTGGGTCCTGAAACAACCCTGTTT pPICZCGPA1
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901 TGAGAAAACATCCTTCATGCTGTTCTTGAACAAGTTCGACATATTTGAGAAGAAAGTTCT pPICZCGPA1
840 TGAGAAAACATCCTTCATGCTGTTCTTGAACAAGTTCGACATATTTGAGAAGAAAGTTCT GPA1
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900 TGACGTTCCGTTGAACGTTTGCGAGTGGTTCAGAGATTACCAACCAGTTTCAAGTGGGAA GPA1
1021 ACAAGAGATTGAGCATGCATACGAGTTTGTGAAGAAGAAGTTTGAGGAGTTATATTACCA pPICZCGPA1
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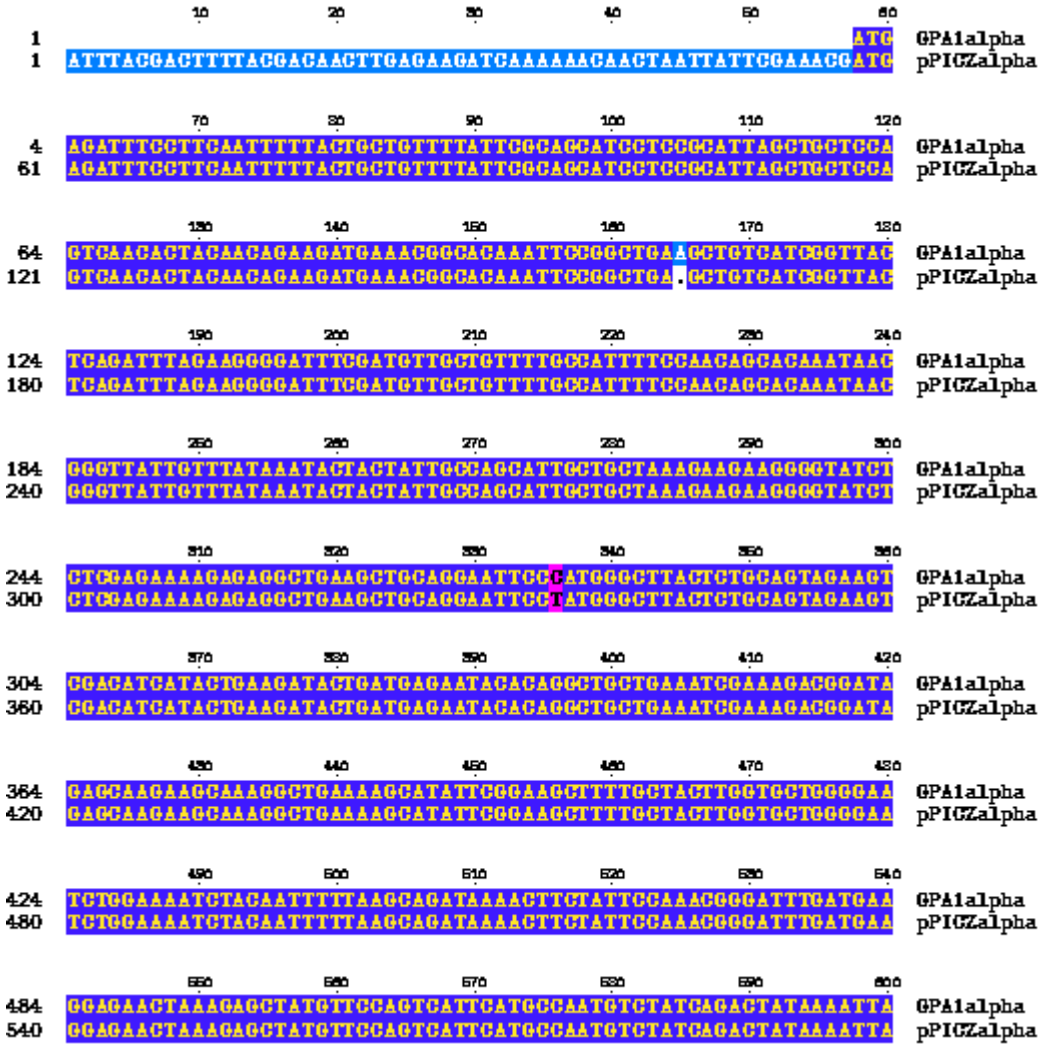
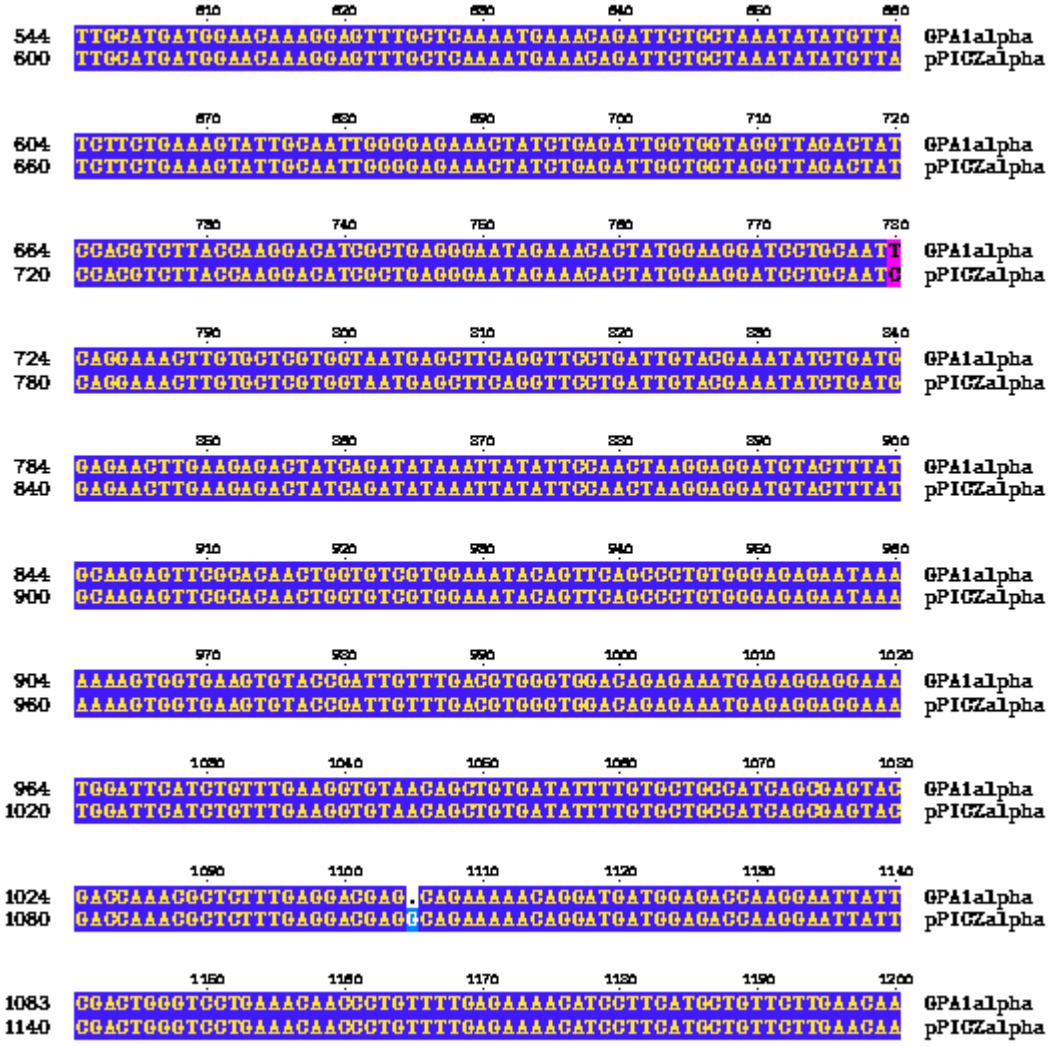
1255 GPA1
1381 AGAT pPICZCGPA1
1255 GPA1
non conserved
similar
conserved
all match

F.4 Sequencing results of GPA1 and pPICZαB+GPA1'-2 construct, with *Eco*RI and *Xba*I restriction enzyme sites at 5' and 3' sites, respectively. Alignments starts at the α-factor signal sequence and ends at the stop codon of polyhistidine tag of pPICZαB.

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600 TTGCATGATGGAACAAAGGAGTTTGCTCAAAATGAAACAGATTCTGCTAAATATATGTTA pPICZalpha
670 680 690 700 710 720
604 TCTTCTGAAAGTATTGCAATTGGGGAGAAACTATCTGAGATTGGTGGTAGGTTAGACTAT GPA1alpha
660 TCTTCTGAAAGTATTGCAATTGGGGAGAAACTATCTGAGATTGGTGGTAGGTTAGACTAT pPICZalpha
730 740 750 760 770 780
664 CCACGTCTTACCAAGGACATCGCTGAGGGAATAGAAACACTATGGAAGGATCCTGCAATT GPA1alpha
720 CCACGTCTTACCAAGGACATCGCTGAGGGAATAGAAACACTATGGAAGGATCCTGCAATC pPICZalpha
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724 CAGGAAACTTGTGCTCGTGGTAATGAGCTTCAGGTTCCTGATTGTACGAAATATCTGATC GPA1alpha
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910 920 930 940 950 960
844 GCAAGAGTTCGCACAACTGGTGTCGTGGAAATACAGTTCAGCCCTGTGGGAGAGAATAAA GPA1alpha
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970 980 990 1000 1010 1020
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1030 1040 1050 1060 1070 1080
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1150 1160 1170 1180 1190 1200
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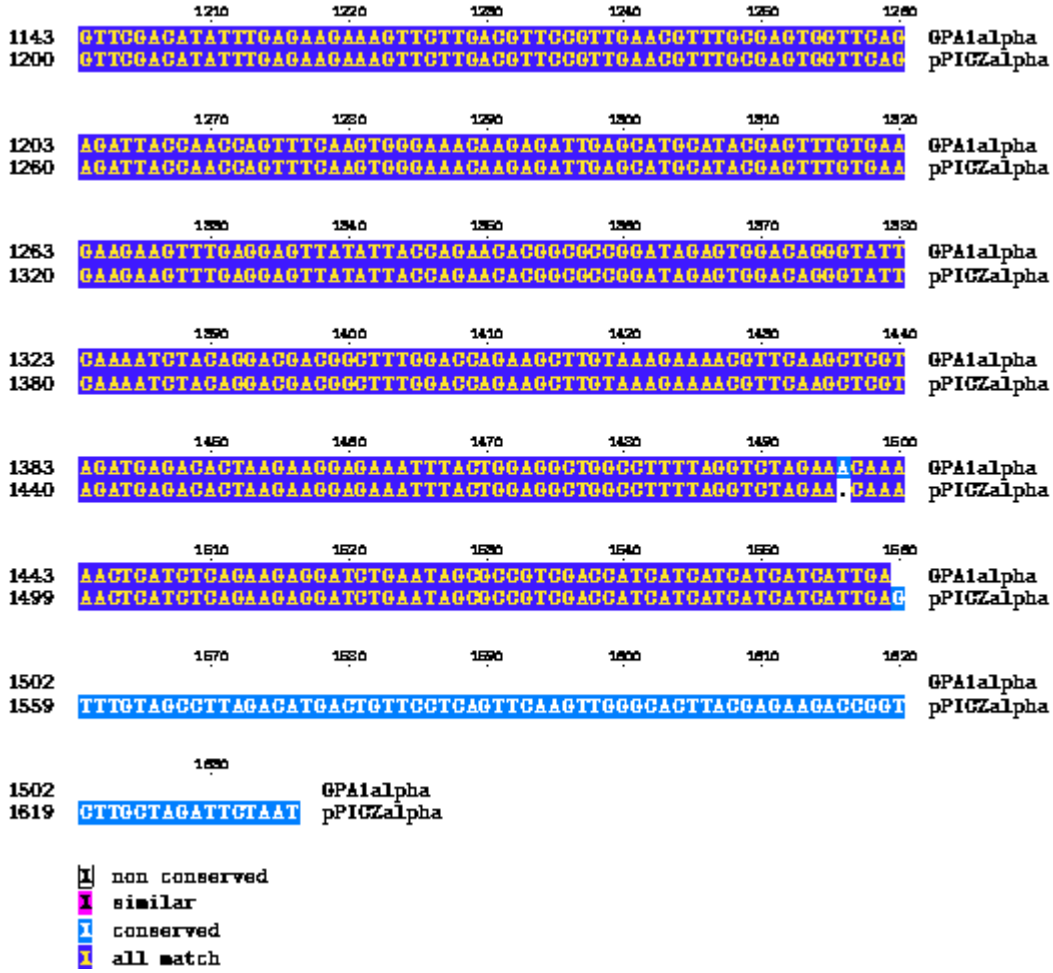
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1270 1280 1290 1300 1310 1320
1203 AGATTACCAACCAGTTTCAAGTGGGAAACAAGAGATTGAGCATGCATACGAGTTTGTGAA GPA1alpha
1260 AGATTACCAACCAGTTTCAAGTGGGAAACAAGAGATTGAGCATGCATACGAGTTTGTGAA pPICZalpha
1330 1340 1350 1360 1370 1380
1263 GAAGAAGTTTGAGGAGTTATATTACCAGAACACGGCGCCGGATAGAGTGGACAGGGTATT GPA1alpha
1320 GAAGAAGTTTGAGGAGTTATATTACCAGAACACGGCGCCGGATAGAGTGGACAGGGTATT pPICZalpha
1390 1400 1410 1420 1430 1440
1323 CAAAATCTACAGGACGACGGCTTTGGACCAGAAGCTTGTAAAGAAAACGTTCAAGCTCGT GPA1alpha
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1450 1460 1470 1480 1490 1500
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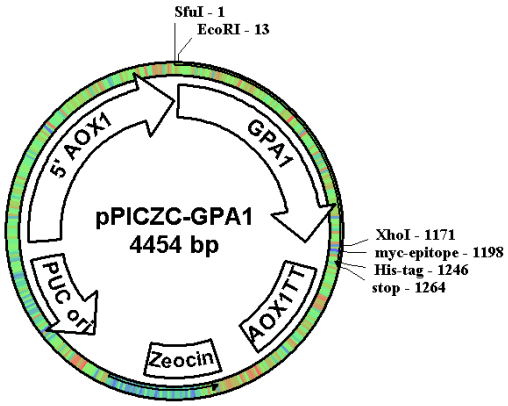
1510 1520 1530 1540 1550 1560
1443 AACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGA GPA1alpha
1499 AACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGAG pPICZalpha
1570 1580 1590 1600 1610 1620
1502 GPA1alpha
1559 TTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGT pPICZalpha
1630
1502 GPA1alpha
1619 CTTGCTAGATTCTAAT pPICZalpha
non conserved
similar
conserved
all match

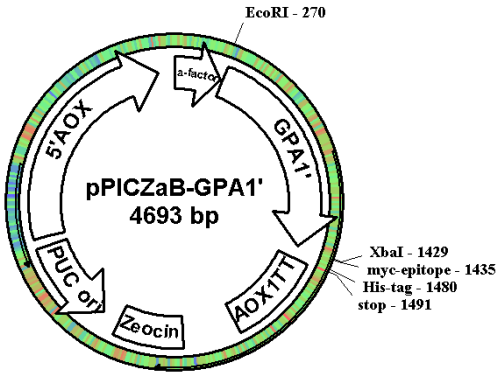
APPENDIX G

CONSTRUCTS

G.1 pPICZC+GPA1



G.2 pPICZαB-*GPA1*'



APPENDIX H

INDUCTION DATA

H.1 KM71H INDUCTION

H.1 Absorption measurements of glycerol (100 ml BMGY) fed KM71H integrants and GS115 Albumin after 20 hours growth

colony	insert	OD_{600}
3	*GPA*1	2
7-2	*GPA*1'	2,9
11'-1	pPICZC	4,08
GS115 Albumin	-	6

H.2 GS115 INDUCTION-1

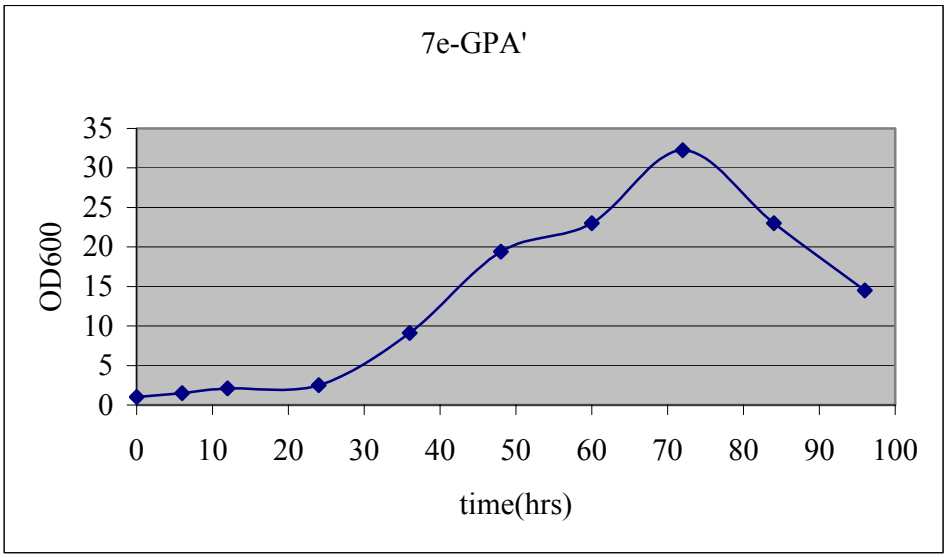
H.2.1 Absorption measurements of GS115 integrants and GS115/ pPICZC/lacZ after 20 hours growth in glycerol (25 ml BMGY). Cultures were induced with a starting OD_{600} of 1.0

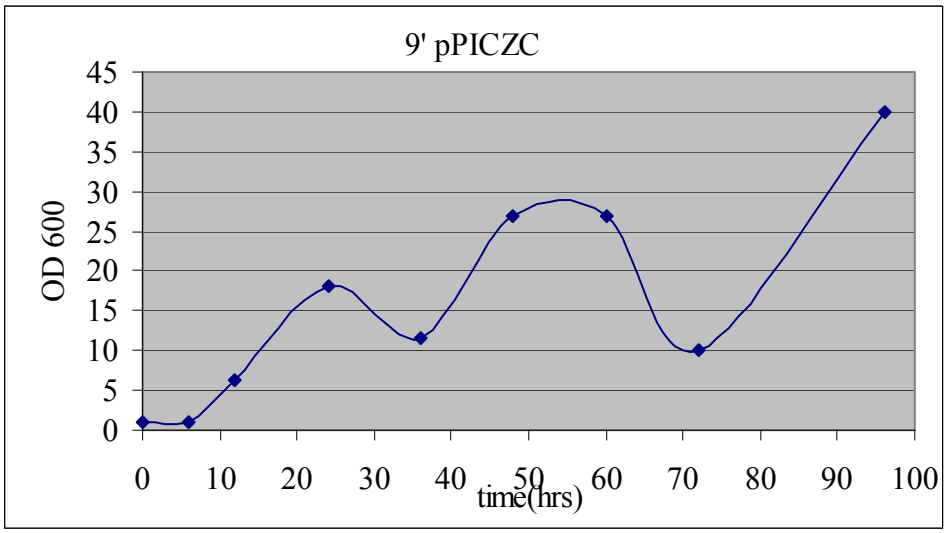
colony	insert	OD_{600}	induction volume
7e-2	*GPA*1'	2,3	60 ml
9'	pPICZC	3,87	96,75 ml
GS115/ pPICZC/lacZ	-	5	125 ml

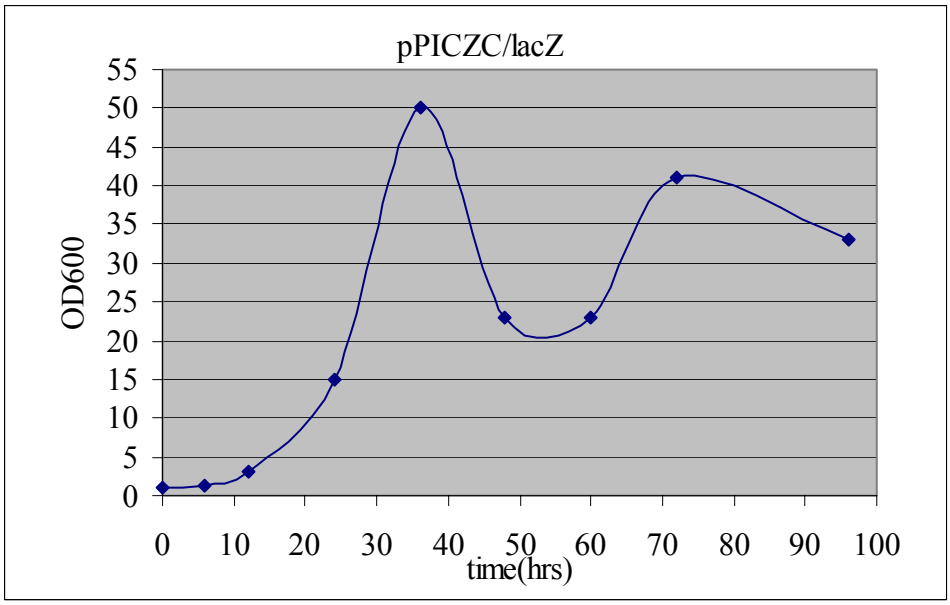
H.2.2 Absorption measurements of GS115 integrants and GS115/ pPICZC/lacZ during induction; at the time points when samples for SDS-PAGE and WESTERN Blot analyses were taken.

time	methanol	7e-2	9'	pPIICZC/lacZ
0	+	1	1	1
6		1,5	1,07	1,4
12		2,1	6,3	3
24	+	2,5	18	15
36		9,1	11,5	50
48	+	19,4	27	23
60		23	27	23
72	+	32,25	10	41
84		23	-	-
96		14,5	40	33

H.2.3 Growth curves of induced strains







H.3 GS115 INDUCTION-2

H.3.1 Absorption measurements of GS115 integrants and GS115/ pPICZC/lacZ after 20 hours growth in glycerol (25 ml BMGY). Cultures were induced with a starting OD_{600} of 1.0

colony	insert	OD_{600}	induction volume
1-1	*GPA*1	2	50 ml
10e	pPICZC	3	75 ml
GS115/ pPICZC/lacZ	-	1,3	50 ml

7 REFERENCES

1. Aharon GS, Gelli A, Snedden WA, Blumwald E, "Activation of a plant plasma membrane Ca^{+2} channel by TGα1, a heterotrimeric G protein α-subunit homologue" *FEBS Letters* 424 (1998) 17-21.

2. Assmann SM, "Heterotrimeric and Unconventional GTP Binding Proteins in Plant Cell Signalling" *The Plant Cell* 14 (2002) S355-S373.

3. Ausubel FM, Brent R., Kingston RE, Moore DD, Smith JA, Seidman JG, Struhl K, "Current Protocols in Molecular Biology" John Wiley & Sons (1994) New York.

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