A. thaliana G PROTEIN γ-SUBUNIT GENE: CLONING, CHARACTERIZATION AND EXPRESSION

by

ÇA□DA□ SEÇK□N

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

Sabanc□ University
July 2003

A. thaliana G PROTEIN γ-SUBUNIT GENE:
CLONING, CHARACTERIZATION AND EXPRESSION

APPROVED BY:

Assoc. Prof. Zehra Sayers …….
(Dissertation Supervisor)

Prof. Dr. Feride Severcan …….

Assoc. Prof. Fatih □zgü …….

Assoc. Prof. Alpay Taralp …….

Prof. Dr. Yuda Yürüm …….

DATE OF APPROVAL: …….

ABSTRACT

The aim of this thesis was to characterize, clone and express *AGG1* gene product for structural studies either on its or own together with beta and alpha subunits.

The *AGG1* gene coding for the *Arabidopsis thaliana* G protein gamma subunit was amplified by PCR and subcloned in *E. coli* for verification and analyses of the cDNA sequence. Following source sequence verification *AGG1* was inserted into different expression vector for overexpression of the recombinant protein. These vectors included pGEX-4T2, pGFPuv, pTrcHis-TOPO and pT7/NT-TOPO. Different E. coli strains including BL21(DE3) and BL21(DE3)pLysS, were tried as host cells. Expression of AGG1-his tag fusion protein using pTrcHis-TOPO in BL21(DE3)pLysS cells was demonstrated. AGG1 protein was detected by Western blotting and coomassie blue staining of polyacrylamide gels.

This study is the first report of AGG1 expression and synthesis of the gene product in a bacterial cell and it provides characterization of different *AGG1* gene containing constructs. Further work is needed for more efficient expression of AGG1 in other host systems.

□□

ÖZET

Bu projenin amacı AGG1 geninin klonlanması, karakterizasyonu ve yapı çalışmaları için tek başına veya alfa ve beta altbirimleri ile birlikte ekspresyonudur.
A. thaliana G proteini gamma alt birimi proteinini sentezleyen AGG1 geni polimeraz zincir reaksiyonu ile çoğaltılıp cDNA sekansının verifikasyonu ve analizi amacıyla *E. coli* hücrelerine klonlanmıştır. Verifikasyonu takiben AGG1 geni, rekombinant proteinin yüksek miktarda üretimi için farklı ekspresyon vektörlerine yerleştirilmiştir. Bu vektörler pGEX-4T2, pGFPuv, pTrcHis-TOPO and pT7/NT-TOPO vektörleridir. Farklı E. coli tipleri olan BL21(DE3) and BL21(DE3)pLysS konak hücre olarak kullanılmıştır. AGG1-his işaretli proteininin sentezi pTrcHis-TOPO vektörü kullanılarak BL21(DE3)pLysS tipi hücrelerde gösterilmiştir. AGG1 proteini Western transfer yöntemiyle ve Komassi boyama ile poliakrilamid jeller üzerinde gösterilmiştir.

Bu çalışma *AGG1* geninin ekspresyonunu ve gen ürünü olan proteinin bakteri hücrelerinde sentezini göstermesi açısından ilktir ve *AGG1* geninin farklı vektörlere yerleştirilerek karakterizasyonu sağlanmıştır. *AGG1* geninin daha verimli ekspresyonunu sağlamak için farklı canlı sistemlerin de denenmesi gerekmektedir.

To my family

□□

□□ **TABLE OF CONTENTS**

□□ **LIST OF FIGURES**

LIST OF TABLES

ABBREVIATIONS

ABA: Abscisic acid

AC: Adenylyl cyclase

AGG1: First identified G_γ gene from *A. Thaliana*

AGG2: Second identified G_γ gene from *A. Thaliana*

AGS: Activator of G protein Signalling

ATP: Adenosine triphosphate

cGMP: Cyclic GMP

C-terminus: Carboxyl terminus

Cys: Cysteine amino-acid

GAP: GTPase activity

G_α: G-protein alpha subunit

G_β: G-protein beta subunit

GDP: Guanosine di-phosphate

G_γ: G-protein gamma subunit

GNEF: Guanine nucleotide exchange factor

GPα1: G_α gene from *A. Thaliana*

GPCR: G-protein coupled receptor

GTP: Guanosine tri-phosphate

GTPase: enzyme converting GTP into GDP

G_α-GDP: G_α bound to GDP, in its active site

Gα-GTP: G_α bound to GTP, in its active site

DAG: Diacylglycerol

EF: Elongation Factor

FR: far-red

IP3: Phosphotidylinositol 3-kinase

NMR: Nuclear magnetic resonance

N-terminus: Amino terminus

PLA: Phospholipase-A

PLC: Phospholipase-C

PhyA: Phytochrome A

Phy B: Phytochrome B

RGS: regulators of G-protein signaling

SA: Salicilic acid

TAIR: *Arabidopsis thaliana* web page (www.arabidopsis.org)

WD40 repeat: Tryptophane-Aspartate repeate consisting of 40 residues

1 INTRODUCTION

Heterotrimeric GTP-binding proteins (G proteins) consist of three subunits; α,β and γ, and function as major signaling elements between G protein coupled receptors (GPCRs) at membranes and intracellular effectors. They have been shown to be key mediators of many important cellular functions such as vesicular transport, multi-drug resistance, cell signaling, phagocytosis, endocytosis, neurotransmitter release, and cell migration to name just a few.

Based on a structural classification, small GTP-binding proteins and elongation factors, along with the Heterotrimeric G proteins make up the three forms of Guanine nucleotide binding proteins.

Small GTP-binding proteins are members of the Ras superfamily, which is divided into Ras, Rho, Rab, Arf and Ran families. Their active form, which binds GTP, interacts with cellular effectors to which they transmit signals of action. The activity of small GTP-binding is controlled by three main classes of proteins, which regulate the alternation between their inactive, GTP-bound form, and their active form: i) Guanine nucleotide exchange factors or GEFs, which activate small GTP-binding proteins by stimulating the dissociation of the tightly bound GDP nucleotide; ii) GTPase Activating Proteins or GAPs, which terminate the signal by stimulating the hydrolysis of GTP; iii) Guanine Nucleotide Dissociation Inhibitors or GDIs, which partition the Rho and Rab subfamilies between membranes and cytosol. Plant homologues of these proteins are classified as Ras, Raf, Arf and ROP.

□□ The protein synthesis elongation factors EF-Tu and EF-G make up the other class of GTP binding proteins. The GTP-bound form of EF-Tu brings the amino acid carrying tRNA to the ribosome while GTP-bound EF-G catalyzes the translocation of amino acid from the A to P site of the ribosome. EF-Tu was the first G-protein whose nucleotide binding domain was solved structurally by X-ray crystallography to yield a structural definition of the GDP-bound form.

□□ Unlike small GTPases, being composed of a single GTP-binding domain, heterotrimeric G proteins are composed of three subunits: a GTP-binding domain (α subunit), β subunit and γ subunit. Under conditions of no ligand binding to GPCRs, the three subunits stay in a complexed form at the interior part of the membrane. Ligand binding to receptor parts accessible from outside the cell results in changes in heterotrimeric G-proteins attached to the inside of the cell plasma membranes. These changes include exchange of an alpha subunit bound guanine diphosphate (GDP) for a guanine triphosphate (GTP). The alpha subunit with bound GTP dissociate from beta-gamma complex and associate, separately, with other intracellular biomolecules including phosphodiesterase, adenylyl cyclase, and phospholipase that regulate the formation of cellular second messengers. Recently, 13 alpha, 7 beta and 2 gamma subunits were identified in plant systems.

□□ The function of a protein is absolutely dependent on its 3-D structure. Hence, efficient crystallization and structure analysis of heterotrimeric G proteins using small angle scattering will be the only way to understand their functional characteristics. GPCRs or G protein-dependent signaling pathways are the targets of a very large number of therapeutically useful drugs. Detailed knowledge about the molecular structure of the elements of G protein signaling pathways should therefore open the gate for the design of novel drugs with increased efficacy and specificity for both humans and plants. However, like every recombinant protein, the protein of interest should be cloned efficiently and expressed under optimal conditions in a suitable organism in high amounts for structural studies and other purposes.

This study is performed in order to clone, express and characterize *Arabidopsis thaliana* G protein gamma subunit (AGG1), for determining its structural features and comparing with mammalian counterparts.

2 OVERVIEW

2.1 G Protein coupled receptors (GPCR)

GPCRs, share the general structure of 7 transmembrane helices which are connected by three intracellular and three extracellular loops (Baldwin *et al*., 1993). These regions provide specific functions to these receptor proteins. The signal transduction mechanism related with the G protein coupled receptors is dedicted to certain stimulating factors including light, Ca^{2+} ions, taste ligands, and small molecules such as amino acids, nucleotides and peptides.

Despite the common structural characteristics, GPCRs have various nucleotide differences among species, resulting in different domains to bind their ligands and activate G proteins. GPRCs are a very diverse group membrane receptor proteins whose members heve distinct sequences and these sequences meke up a relatively high percentage of the total genome of most organisms; i.e. 5% in C. elegans (Joel and Jean, 1999) and 1% in human (Marinissen *et al*., 2001).

As shown in figure 1.1, among the three cytoplasmic loops, I2 and I3 loops are critical in G protein recognition and activation. Based on the studies of rhodopsin structure, the change from inactive to active state is achieved with a change in the relative orientation of transmembrane helices 3 and 6, which unmasks G protein binding sites (Joel and Jean, 1999). This is necessarily a consequence of ligand binding; and association of GPCR in this conformation with G proteins promotes the exchange of GDP for GTP at G alpha subunit and triggers the separation of βγ dimer from α (Hur *et al*., 2002). Also a ligand-induced conformational change at the COOH terminus of alpha

subunit may result in a reduced binding and dissociation from the complex (Yang *et al*., 1999). Recently, homo- or hetero-dimerization of GPCRs via their sixth transmembrane helices has been proposed instead of one receptor-one G protein view. This dimerization could be important for activation of G proteins. Additionally, palmitoylations at conserved Cys residues and phosphorylations at Ser residues could direct and modify the coupling specificity of GPCRs (Joel and Jean, 1999).

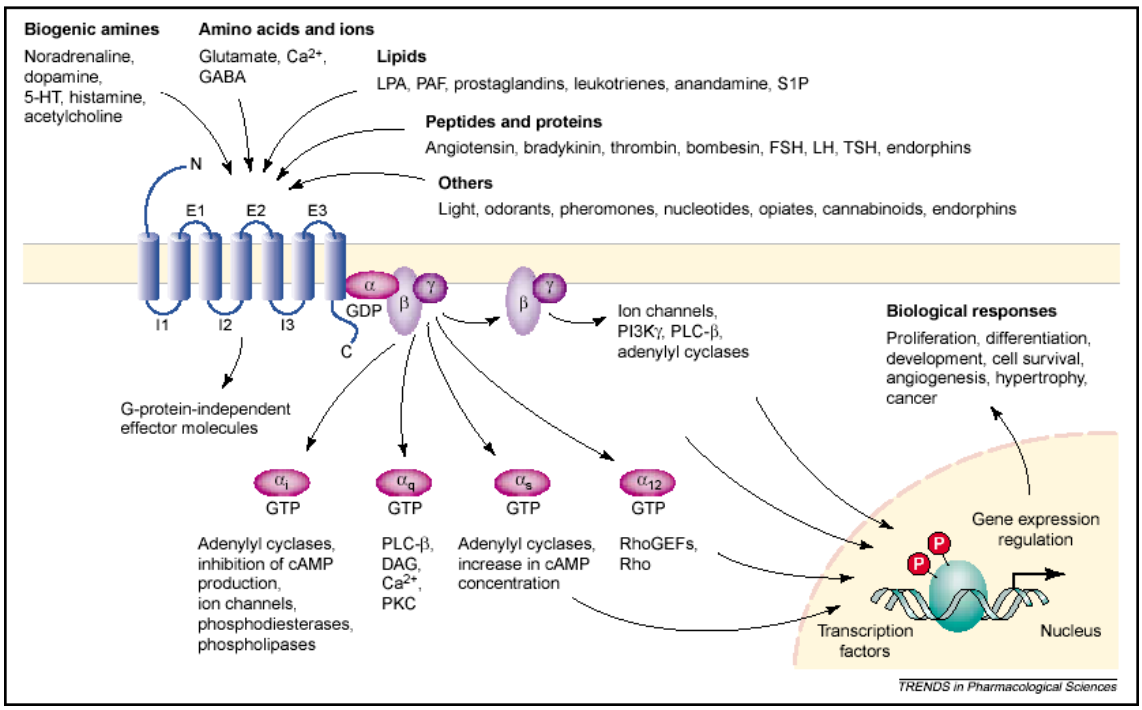


Figure 2.1 GPCR signalling pathways (Marinissen *et al*., 2001)

□□ In plants, specifically *A. thaliana*, there is no receptor complex that is proved to interact directly with G proteins. However, there are some candidates among which MLO1 is the most studied candidate, despite the mechanism of action is unknown. In addition, GCR1 shares some sequence identities with the animal GPCR members rhodopsin7serotonin family (Alan, 2002).

2.2 Receptor Independent G protein signalling

Three proteins as the Activators of G protein Signalling (AGS1, AGS2 and AGS3) were reported in *Saccharomyces cerevisae*, to function on heterotrimeric G protein signaling in the absence of a receptor protein. Although the starting stimulus or the mechanism of AGS1 activation, if any, is still to be solved; yeast protein assays show that AGS1 and 3 preferentially bind to the α subunit while AGS2 interacts with the βγ dimmer (Mary *et* al., 2001). Moreover, AGS3 has been studied to be a guanine dissociation inhibitor (GDI) which means that in interacts with the GDP-bound form of G_α to dissociate from βγ (De Vries *et al*., 2000). No plant counterpart of these proteins has been identified yet.

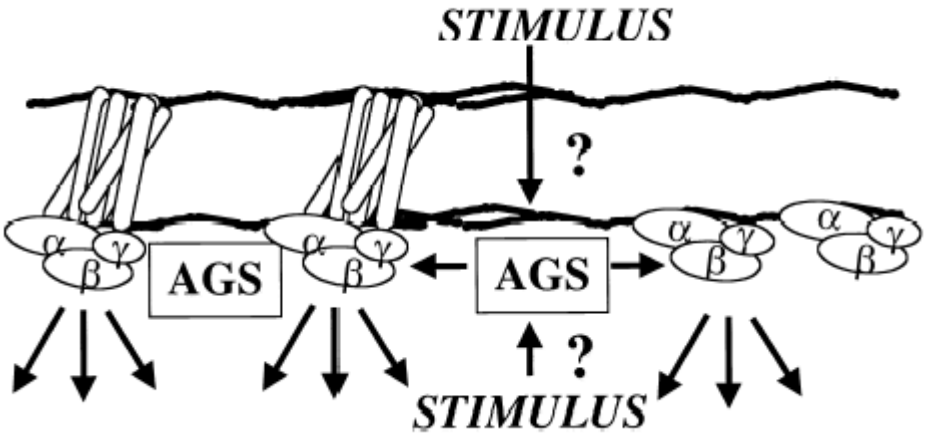


Figure 2.2 Schematic representation of AGS proteins of yet unidentified signal processing (Cismowski *et al.,* 2001)

2.3 Small GTP binding Proteins

Ras superfamily is the group of small monomeric GTP binding proteins, consisting of Ras, Rho, Rab, Arf and Ran families. Small GTPases recruitment can be controlled by the action of Guanine Nucleotide Dissociation Inhibitor (GDI). GDI

activity in turn can be activated by cytoskeletal proteins. Ligation of growth factor receptors or integrins leads to recruitment of Guanine Nucleotide exchange factor (GEF) by post-translational modifications or binding phospholipids. Signal transduction by activated Ras proteins is likely involve translocation of proteins to the membrane compartment. Termination of signal transmission is also controlled at multiple levels, including targeted degradation of GEFs through ubiquitination and stimulation of GTP hydrolysis by GTPase activating proteins (GAPs) (Takai *et al*., 2001).

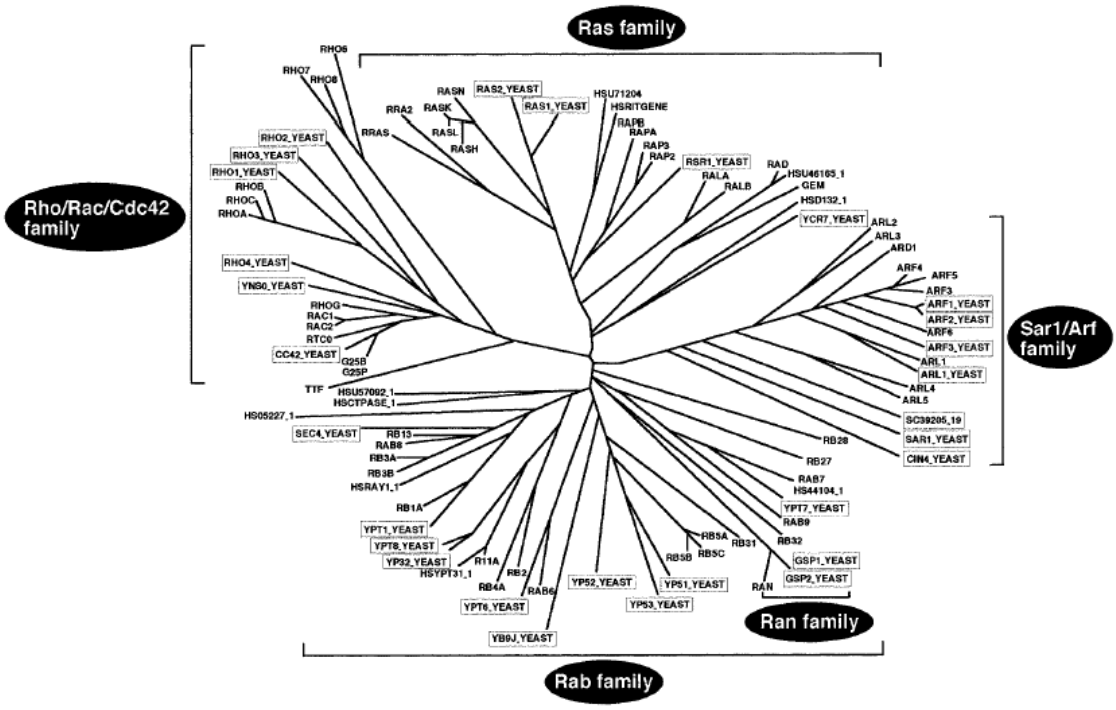


Figure 2.3 Small G protein superfamily (Takai *et al*., 2001)

□□ The complete activation of Ras-related proteins occurs via either the ARF-Gea2p or Ras-SOS mechanisms. As the result, GTP should enter the nucleotide binding site and dissociate the GEF. In both complexes, due to the open nucleotide site through the solvent up to the α-phosphate group, GTP is allowed to enter by its GMP moiety (Cherfils and Chardin, 1999). The return of Ras to its GDP-bound form makes Raf to dissociate, activating MEK and MAP Kinase, to regulate gene expression by phosphorylating transcription factors inside the nucleus (Hilgenfeld, 1995).

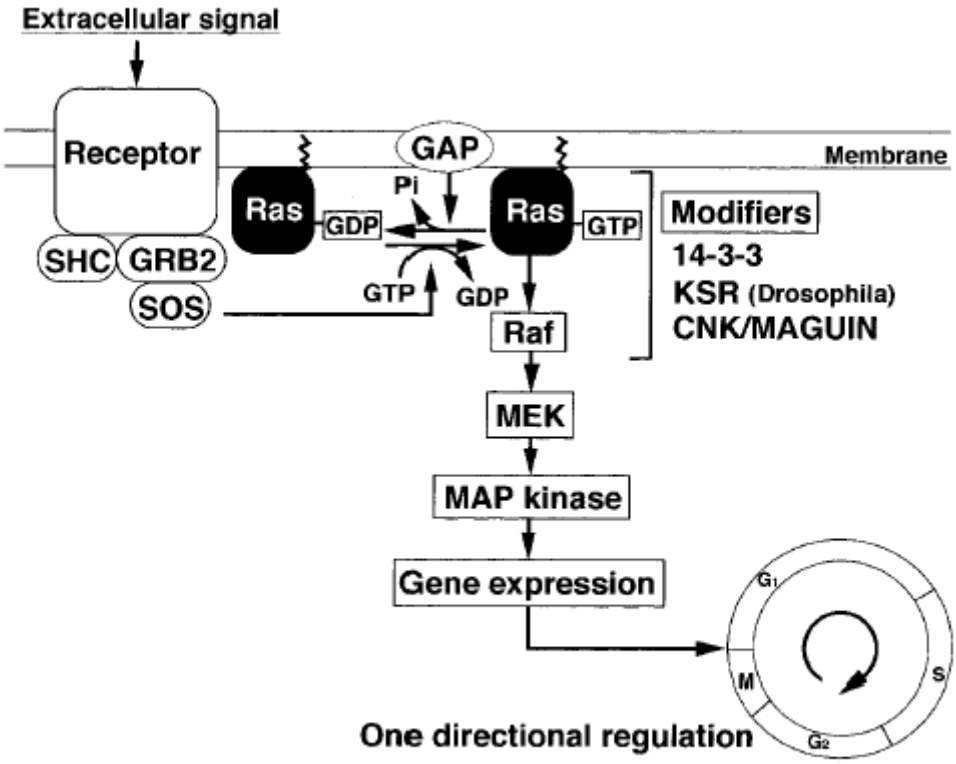


Figure 2.4 Signal Transduction downstream from Ras (Takai *et al*., 2001)

2.4 Elongation Factors

The initiation of translation machinery requires participation of elongation factors EF-Tu, EF-Ts and EF-G, among which EF-Tu and EF-G are small GTP binding proteins. EF-Tu-GTP binds and delivers an aminoacyl-tRNA to the A site on the ribosome. As the aa-tRNA is delivered by EF-Tu to the A site on the ribosome, GTP is hydrolysed to GDP+P_i. A large conformational change occurs due to GTP hydrolysis releasing EF-Tu, which in turn leads to repositioning of the aa-tRNA to promote peptide bond formation. Translocation of the ribosome relative to mRNA involves the GTP binding protein EF-G, attaching to the A site. This EF-G-GTP binding pushes the tRNA with attached nascent polypeptide from the A site to the P site (Sprang, 1997).

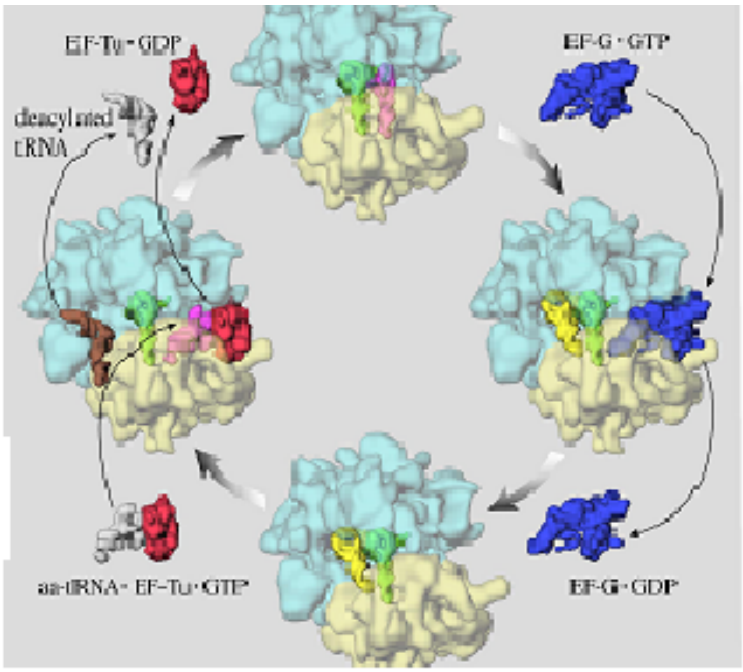


Figure 2.5 Mechanism of EF activation (www.lebs.cnrs-gif.fr)

2.5 Heterotrimeric G proteins

2.5.1 General Information

□□ Heterotrimeric GTP binding proteins (G proteins) play a central role in many types of transmembrane signalling pathways by transmitting information from receptors to effector molecules. G proteins are composed of α, β and γ subunits having molecular weights of ~45 kDa, ~35kDa and 7-10 kDa, respectively (Danner *et al*., 1996). Under physiological conditions, the β and γ subunits are tightly associated with each other and are present as a dimer. The α subunit could be described as the major active site of the heterotrimer, due to participating in a greater number of intracellular pathways than the βγ dimer.

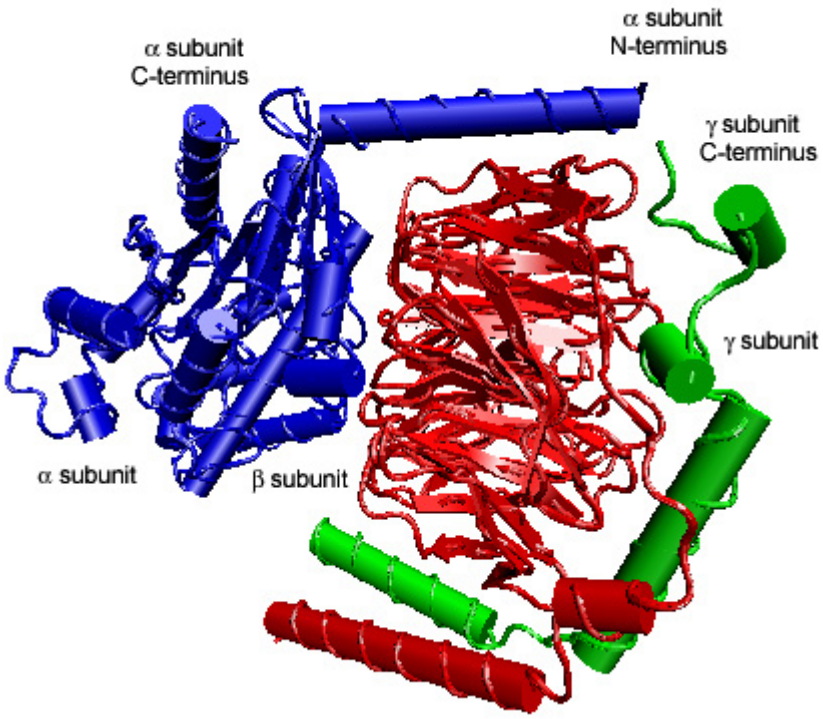


Figure 2.6 Heterotrimer

□□ The activation of the heterotrimer occurs by the binding of the ligand to a GPCR protein, generally, and consequently with the reorientation of the domains of receptor protein resulting in a different interaction power with the heterotrimer. This rearrangement leads to an exchange of GDP with GTP on the α subunit and dissociation of α from βγ. As much as 23 α, 6 β and 12 γ subunits have been identified in animals (Alan, 2002).

□□ In contrast to mammals and invertebrates, *A. thaliana* genome contains only one G_{α} (GPA1), one G_{β} (AGB1) and two G_{γ} (AGG1-AGG2) (TAIR Homepage). The existence and possible roles of each subunit together with the similarities with mammalian counterparts will be discussed later.

2.5.2 G protein α subunit

There are 4 classes of mammalian G protein alpha subunits, consisting altogether of at least 23 proteins. These classes are: G_s (s-for stimulatory) activating adenylyl cyclase; G_i (i-for inhibitory) inhibiting adenylate cyclase; G_q, for activating phospholipase C (PL-C) and G_{12}, for the regulation of small GTPases (Ma, 1994). The alpha subunits have common structural domains including a Ras-like domain, an α-helical domain which determines GDP release and an Asp-Glu rich loop. The latter two are the ones that do not exist in small GTP binding proteins (Assmann, 2002). Until now, only two of these mammalian G_α subunits have been described. These stuructures; $G_t\alpha$ and $G_i\alpha_1$ are both solved at their GTP and GDP bound states together with their regulators RGS4. $G_t\alpha$ transduces the signal from rhodopsin to its effector molecule cGMP phosphodiesterase while $G_i\alpha_1$ functions in the downregulation of adenylyl cyclase (Bohm *et al*., 1997).

The guanine nucleotide is kept at the contact of two major domains of the alpha subunit; the Ras-like GTPase and the helical domain. There are three switches in the GTPase domain which are conformationaly sensitive to the GTP or GDP bound state and that form the interface between the subunit and βγ part. When GTP is bound, the switch regions are kept in contact with the nucleotide's third phosphate group. In the GDP-bound state, the switches are flexible, reflecting their direct role in the α-βγ interaction (Bohm *et al*., 1997).

The G protein alpha subunit interacts with a large number of effector molecules including adenylyl cyclase, cGMP phosphodiesterase, phosphoinositide 3-kinase, phospholipase D, Na^+/H^+ exchanger protein, transcription factor TUBBY, and potassium, calcium, chloride and sodium channels (Assmann, 2002).

The Arabidopsis genome contains only one identified alpha gene (GPA1). Also, other homologs of GPA1 from several different plant species have been cloned and expressed, which show sequence identities in the range of 34-42% with the mammalian counterparts (Plakidou-Dymock *et al*., 1998).

A major effect of GPA1 in plants is related with its role in cell division. It is reported to be highly expressed in meristematic tissues and the regulation of cell division via alpha subunit is performed by its binding to some other downstream proteins, specifically PLA $_2$, which is known to regulate cell proliferation in many cell types (Capper and Marshall, 2001).

Studies with tomato plants reveal that G protein alpha subunit may function, although not proved structurally, on the regulation of ion channels. The open state probability of Ca^{2+} channels in the membrane was shown to increase in TGα1 containing and GTPase deficient membranes (Aharon *et al*., 1998).

K^+ and Ca $^{2+}$ channels are among the most important downstream effectors. In higher plants, regulation of stomatal opening is dependent on the concentration of K^+, Cl^-, $malate^{2-}$ and sucrose in the guard cells. Abscisic acid is a phytohormone that helps water conservation in plants by decreasing the aperture of the stomatal pores in epidermis and hence decreasing water loss. Wang *et al*., 2001 showed that, GPA1 mutant *A. thaliana* lacks both the ABA inhibition of the inward K^+ channels and pH-independent activation of ion channels. Therefore, water loss in these GPA1 mutants is greater when compared with wild type plants.

2.5.3 G protein β subunit

Six mammalian isoforms of the G protein beta subunit have been described to date. The most important structural feature of β is the overall 7 tandem repeats of WD-40 motif and a conserved Trp-Asp (WD) motif (Assmann, 2002). Although this could be defined as a general characteristic of the beta family, there are many other proteins which contain the WD-40 motif and are not even related with the G protein β subunits. Constitutive Photomorphogenic1 and Transparent Testa Glabra1 are only two examples of this (Lease *et al*., 2001). But overall, the general 3-D structure of the β family of

proteins is defined as an N-terminus α-helical domain which is foollowed by a short stretch of polypeptide chain linking it to the WD-40 motif (Hamm, 1998).

There is one G protein beta subunit derived from the *A. thaliana* genome (AGB1) which shows 42% similarity to the mammalian G $_{\beta}$ subunits 2 and 3(Weiss *et al*., 1994). It is known from structural studies that animal G_{β} subunits are anchored to the cell membranes via Gγ subunits. It would be a disaster to think about a different mechanism, other than the tight association between β and γ subunits, for the plants but up to date there is no structural study revealing the type and degree of association between β and γ subunits. However, in some of the studies it has been shown that G_{β} is a membrane-associated protein in plants. G_{β} was highly rich in microsomal pellet, and when these microsomes were treated with salts and detergents G_{β} was finally localized on the membranes, attaching via hydrophobic interactions (Obrdlik *et al*., 2000).

One mutant allele of AGB1, *agb1-1,* constructed by Lease *et al*. resulted in a large number of defects in *A. thaliana* including short fruits, rounded leaves and shortened floral buds, demonstrating the possible role of the beta subunit on plant development.

2.5.4 G protein γ subunit

Among the 12 mammalian G protein γ subunits identified, a small size ranging between 7-11 kDa,a prenyl group binding site on the C-terminus (CAAX box) and an N-terminus coiled coil domain can be counted as general structural features. The most important point to be considered is that, β and γ are normally found as a dimer and they cannot be separated except highly denaturing conditions. Therefore, the overall conformation of the dimer could give an important view about the stuctures separately. G_{γ} is studied to form a coiled coil part with the N-terminus of G_{β} and continues extending around the β-propeller. This conformation makes the outer part of the propeller positively charged, which is adjacent to the acyl-modificated part of G_{γ}, together making up the membrane-anchoring region of the heterotrimer (Bohm *et al*., 1997).

□□ The first plant G protein γ subunits were identified, named AGG1 and AGG2 , in 2000 and 2001 respectively by Mason and Botella. These subunits show 48% sequence identity to each other and 24-31% sequence identity to non-plant counterparts. However, the common functional domains are almost identical among these, including C-terminal and N-terminal regions. AGG1 and AGG2 are shown to interact with *A. thaliana* β subunit (AGB1) using both yeast two-hybrid and in vitro binding assays. Southern and Northern blotting studies sh owed that AGG1, just like AGG2, is a single-copy gene, being expressed differentially in specific tissues.

□□ TGB1, which is the Tobacco G protein β subunit, to be used as bait, was cloned into pAS-CYH vector and transferred into yeast. Retransformation was performed with the *A. thaliana* yeast two hybrid library. Positive colonies possessing β-Galactosidase activity were isolated and a secondary screening was done by transforming yeast with the library plasmid plus TGB1-pASCYH construct and the library plasmid plus another plasmid being used as negative control. One positive clone, which is the AGG1 was obtained several times, at the end of yeast two-hybrid protocols.

□□ For in vitro binding assay, recombinant vector containing AGG1-GST fusion sequence was constructed, expressed in *E. coli* and purified. On the other hand, AGB1 RNA was synthesized, and translated using ^{35}S-methionine. The product was added to the matrix containing AGG1-GST and GST. Strong binding of AGB1 to AGG1-GST fusion protein together with no binding to single GST reveals interaction of β and γ subunits (Mason and Botella, 2000).

2.6 Bγ-linked effectors

□□ The βγ dimer activates a wide range of effector molecules inside the cell. These molecules include some isomers of adenylyl cyclase, phospholipase C, phospholipase A_2, phosphoinositide 3-kinase and K^+ channels.

Adenylyl cyclase is an intracellular enzyme that catalyzes the formation of cAMP using Mg^{2+} bound ATP. Sharing a common domain of two membrane spanning regions of six α-helices which connect 40-kDa cytoplasmic domains; nine different isofor ms of adenylyl cyclases have been cloned and expressed to date, designated as AC I to IX. All of the isoforms are subject to activation by $G\alpha_s$ additionally, type I is inhibited while type II and IV are activated by βγ subunits (Morris and Malbon, 1999). However, both activation and inhibition requires high concentrations of βγ (Tang *et* a*l*., 1992).

Inositol lipid-specific PLCs consist of three groups β, γ and δ. Among these, the four PLC- β isoenzymes are potential targets for G proteins and βγ dimer can activate all of these isozymes. PLC-β forms secondary messengers such as phosphaditylinositol 3-kinase (IP_3) and diacylglycerol (DAG) by hydrolyzing inositol lipids, which respectively, controls the intracellular levels of Ca^{2+} and protein kinase C (PKC), to progress in cell growth and cell fate. For PLC-β_3, the α_q and βγ subunits are equally effective while for the other member of PLC-β family, α_q is 50 to 100 fold more effective than βγ. The lower capacity of βγ subunits when compared with the α subunits may play role as a level of selectivity in receptor regulation of PLC-β enzymes. Two structurally conserved regions are observed in PLC-β enzymes suitable for subtrate binding and catalysis, named X and Y domains. Although the complete mechanism between PLC-β and $G_\alpha/G_{\beta\gamma}$ has not been defined structurally, The X and Y regions that comprise the NH2 terminus, the inter X-Y region and the COOH terminus functions in PLC-β G protein coupling (Morris and Malbon, 1999).

Phosphoinositide 3-kinases (PI 3-kinases) are a group of ATP-dependent enzymes that catalyze the phosphorylation of three myositol containing phospholipids phosphatidylinnositol, phosphatidylinositol 4-phosphate and PIP_2 forming phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-triphosphate, respectively. PI 3-kinases perform function in many intracellular processes including receptor mediated signaling, protein trafficking and mutagenesis. Many PI 3-kinases studied in mammalian cells are reported to be

activated by G protein βγ subunits. Recently, p85 associated PI 3-kinase found in human platelets is shown to be activated by βγ subunits (Carter and Downes, 1992).

□□ Phospholipase A_2 (PLA_2) which catalyzes the rate limiting step in the synthesis of prostanoids, the release of arachidonic acid from plasma membrane lipids, are activated by the $G_{\beta\gamma}$ subunit in retina (Jelsema and Axelrod, 1987).

□□ K^+ concentration and the pumping of K^+ channels are important aspects of contraction of heart muscles. Hyperpolarization leads to slowing in the contraction rate of the heart. Different studies suggested that the K+ channel regulating the contraction rate of heart is directed by G protein dependent mechanisms. Addition of $G_{\beta\gamma}$ resulted in up to 500 fold increase in channel activity (Kim *et al*., 1989; Wickman *et al*., 1994).

2.7 Plant G Protein Classification

Gene	Species	Classification	Reference
GPA1	Arabidopsis	Gα	Ma et al., 1990
TGA1	Tomato	Gα	Ma et al., 1991
LjGPA1	Lotus	Gα	Poulsen et al., 1994
RGA1/D1	Rice	Gα	Ishikawa et al., 1995; Seo et al., 1995
SGA1	Soybean	Gα	Kim et al., 1995
SGA2	Soybean	Gα	Gotor et al., 1996
NtGPα1	Tobacco	Gα	Saalbach et al., 1999
NtGA2	Tobacco	Gα	Ando et al., 2000
LGPα1	Lupin	Gα	Kusnetsov and Oelmueller, 1996b
AfGα1	Wild oat	Gα	Jones et al., 1998

PGA1, PGA2	Pea	Gα	Marsh and Kaufman, 1999
SOGA1	Spinach	Gα	Perroud et al., 2000
NPGPA1	*Nicotiana plumbaginifolia*	Gα	Kaydamov et al., 2000
AGB1	Arabidopsis	Gβ	Weiss et al., 1994
ZGB1	Maize	Gβ	Weiss et al., 1994
TGB1	Tobacco	Gβ	Kusnetsov and Oelmueller, 1996a
RGB1	Rice	Gβ	Ishikawa et al., 1996
AfGβ1	Wild oat	Gβ	Jones et al., 1998
AfGβ2	Wild oat	Possible Gβ	Jones et al., 1998
NPGPB1	*Nicotiana plumbaginifolia*	Gβ	Kaydamov et al., 2000
AGG1	Arabidopsis	Gγ	Mason and Botella, 2000
AGG2	Arabidopsis	Gγ	Mason and Botella, 2001
GCR1	Arabidopsis	Potential heterotrimeric G protein receptor	Josefsson and Rask, 1997; Plakidou-Dymock et al., 1998
MLO	Barley	Potential heterotrimeric G protein receptor	Devoto et al., 1999
AtXLG1	Arabidopsis	Extra large GTP binding protein	Lee and Assmann, 1999
PsDRG	Pea	Developmentally regulated G protein	Devitt et al., 1999
AtDRG	Arabidopsis	Developmentally regulated G protein	Etheridge et al., 1999; Devitt et al., 1999
RDH3	Arabidopsis	(putative GTP-binding protein)	Schiefelbein and Somerville, 1990
ATGB1	Arabidopsis	GTP-binding protein	Biermann et al., 1996
fw2.2/ORFX	Tomato	(putative GTP-binding protein)	Frary et al., 2000

Table 2.1 Plant G protein designations *(Assmann SM, 2002).*

2.8 Unconventional GTP binding proteins in plants

In plants there are some other GTP binding proteins having yet unidentified domains. Very large G proteins including AtXLG1, AtXLG-like and G_{α}-putative can be considered as the first group, among which only AtXLG1 gene is the characterized one. Although AtXLG1 is shown to encode an instable protein in recombinant studies, it possesses a helical and a C-terminal G_{α}-like domain in addition to an Asp/Glu rich loop. Another example of Arabidopsis large proteins can be RHD3, a 89 kDa protein containing two regions common to GTPases, thougt to be effective in cell proliferation in the shoot and root parts of the plant (Assmann, 2002). Two other G proteins, smaller than the mentioned ones above are *A. thaliana* ATGB1 (Biermann *et al*., 1996) and tomato ORFX (Frary *et al*., 2000).

2.9 Lipid modifications

Heterotrimeric G protein α and γ subunits are subject to lipid modifications at their specific regions. No G protein β subunit has been identified to contain lipid modification yet. G_{α} subunits are palmytoylated and/or myristoylated while Gγ subunits are isoprenylated by either farnesyl or geranylgeranyl addition (Houslay, 1991).

All of the G protein α subunits cloned to date are shown to have specific binding sites for palmitate or myristate, or both. Myristoylation, which is generally called N-myristoylation occurs at the glycine (generally Gly-2) residue of the very N-terminus of the alpha subunit, as a co-translational addition. Myristate is a 14-carbon saturated fatty acid. The amide bond, linking a myristate to glycine residue at the N-terminus is thougt to be an irreversible binding and seen in α_i family (i1, i2, i3, o, z, t) of the α subunits. Specifically, α_t subfamily in retinal photoreceptor cells contains three additional hydrophobic fatty acylations, which could be an example of tissue specific differences, resulting from different fatty acid pools of the cells. In addition to N-myristoylation, all G_{α} subunits except the α_t subfamily are palmitoylated at their cysteine residues in the N-

terminus. Palmitate is a 16-carbon saturated fatty acid and binding is thougt to be reversible (Wedegaertner *et al*., 1995). Recently, a second palmitoylation at glycine residue, in the N-terminus adjacent to the palmitoylated Cys, has been identified by Kleuss and Krause by peptide mass fingerprinting. The lipid modification hence occurs in G_{α} subunits as both myristoylation and palmitoylation most of the time and the sequence M-G-C is studied to represent the signal for both acylations (Sprang, 1997; Wedegaertner *et al*., 1995).

□□ Lipid modifications provide essential activities for the alpha subunits. Myristoylation and palmitoylation results in the membrane anchoring of α. Palmitate, containing a 2-C more tail region than myristate resulting in a greater hydrophobicity, confers a stronger association with membrane phospholipids (Wedegaertner *et al*., 1995). Myrsitate, probably is not sufficient for a protein membrane linkage and requires the involvement of positive charges and other protein-protein interactions while palmitate has an adequate binding energy to anchor the alpha subunit to the membrane (Peitzsch *et al*., 1993). Fatty-acylated α subunits also have higher affinity protein-protein interactions fro the other G protein subunits. Thioacylation (palmitoylation at the Cys residue) increases the effective linkage of $G_{s\alpha}$ to $G_{\beta\gamma}$ to five-fold, together with inhibiting the interaction of it with the G protein regulators (Manahan *et al*., 2000).

□□ Heterotrimerc G protein γ subunits are isoprenylated by the addition of either a 15-carbon isoprenoid farnesyl (only in the case of γ_1 specific to retina) or a 20-carbon isoprenoid geranygeranyl. The addition occurs to the Cys residue of the CAAX box, which is common in all γ subunits. After isoprenylation, three aminoacids falling to the C-terminal site of modified point are removed and the new C-terminus is carboxymethylated (Wedegaertner *et al*., 1995).

□□ As in the case of α subunits, the lipid modifications in γ subunits help membrane anchoring and play role in protein-protein interactions. The farnesyl group added to the γ subunit may interact directly with an integral membrane protein, or a transmembrane receptor. Geranygeranyl group, with a longer hydrophobic tail, is more eligible for that function, than farnesyl (Manahan *et al*., 2000). Expression of mutant, non-prenylated γ subunits in cultured cells resulted in βγ dimers located in cytosolic rather than

membrane fractions (Simonds, 1994). The yeast Gγ subunit Ste18p is shown to be farnesylated on its C-terminal Cys group. In the absence of prenylation the protein is nonfunctional. However, replacement of the C-terminal lipidation region with a transmembrane domain results in a functional protein, which argues with the membrane anchoring funtion of lipid modification. Indeed, isoprenylation is considered to have a minor effect, if any, on the membrane localization of gamma subunits, when compared with the interaction of coiled region of gamma with membrane phospholipids. Additionally, carboxymethylation also contributes to the membrane association of the βγ dimer, by neutralizing the negatively charged C-terminus of γ (Wedegaertner *et al*., 1995). It is stated by Silvius and l'Heureux that isoprenylation of γ isrequired for appropriate interaction of βγ dimer with the α subunit but the structural aspect is not well understood.

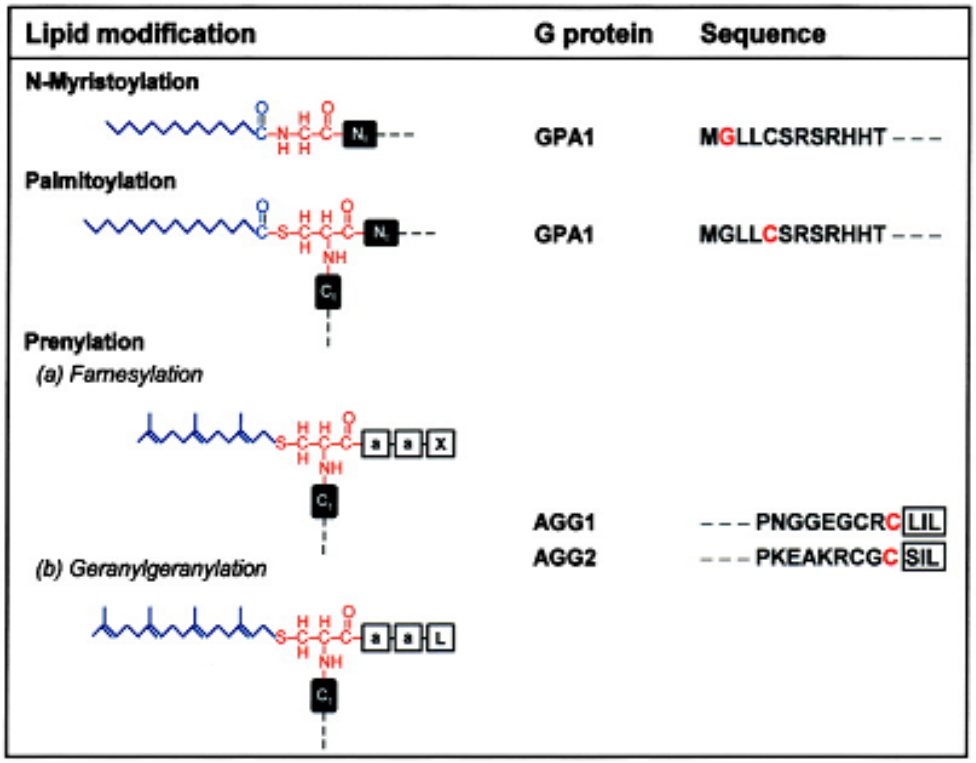


Figure 2.7 Lipid modifications of G protein α and γ subunits (Assmann, 2002)

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Commercial Kits

PCR Core System II (Promega)

pGEM® -T and pGEM® -T Easy Vector Systems (Promega)

Quiaquick® PCR Purification Kit (250) (QIAGEN**)**

Quiaquick® Gel extraction Kit (250) (QIAGEN)

Quiaprep® Spin Miniprep Kit (250) (QIAGEN)

QUIAGEN® Plasmid Midi Kit (100) (QIAGEN)

TOPO® XL PCR Cloning Kit (Invitrogen)

TOPO® TA Cloning Kit (Invitrogen)

TOPO® T7 Expression System (Invitrogen)

TOPO® pTricHis Expression System (Invitrogen)

3.1.2 Chemicals

All chemicals were supplied by Fluka (Switzerland), Merck (Germany), Riedel de Häen (Germany), and SIGMA (USA).

3.1.3 Vectors

AGG1-pBS was kindly donated by Dr. Botella (Universtiy of Queensland, Australia)

pUC18 (Fermentas)

pGEM®-T Easy (Promega)

pGEX-4T2 (Amersham Pharmacia Biotech)

pGFPuv (Clontech)

pCR® II- TOPO® (Invitrogen)

TOPO-T7 (Invitrogen)

TOPO-pTricHis (Invitrogen)

3.1.4 Cells

Different *E. coli* strains containing TOP10, XL1Blue, BL21 (DE3), BL21(DE3)pLysE, Rosetta(DE3), Rosetta(DE3)pLysS were kindly provided by EMBL, Hamburg

BL21-CodonPlus® (DE3)-RIL Competent Cells (Strategene)

TOP10 F' (Invitrogen)

BL21 (DE3) pLysS (Invitrogen)

3.1.5 Enzymes

3.1.5.1 Restriction Enzymes

RE	Cutting Site
BamH l	G▼GATCC
EcoR I	G▼AATTC
Hind III	A▼AGCTT
Sac I	GAGCT▼C

Xho I	C▼TCGAG

Spe l	A▼CTAGT

Hinc II	GT(T/C)▼ (A/G)AC

Kpn I	GGTAC▼C

Nco I	C▼CATGG

Nde l	CA▼TATG

Nhe I	G▼CTAGC

3.1.5.2 DNA Polymerases

Tag Polymerase(Promega)

DNA Polymerase I Klenow fragment for “Fill-in” (Promega)

3.1.5.3 Calf Intestine Alkaline Phosphatase for 5’ dephosphorylation (Promega)

3.1.5.4 Ligase

T4 DNA Ligase (Fermentas)

LigaFast™ Rapid Ligation System (Promega)

3.1.6 Primers

Primers were designed according to the coding sequence of *AGG1* (www.arabidopsis.org). Primers with restriction enzyme sites were purchased from SEQLAB (Germany) and SIGMA (USA).

3.1.7 Buffers and Solutions

Standard buffers and solutions used in cloning and electrophoresis were prepared according to Sambrook J., 2001.

3.1.8 Equipment

All equipment used in this study can be found at Appendix D.

3.2 Methods

3.2.1 Bacterial Cell Cultures

□□ Bacteria were grown for 10-18 hours in LB liquid medium with or without selective reagents prior to application depending on cell type and plasmid copy number. All incubations were performed at 37ºC, with 300 rpm shaking. Miller's LB Agar solid medium was used containing selective reagents for the growth of bacteria after transformation protocols.

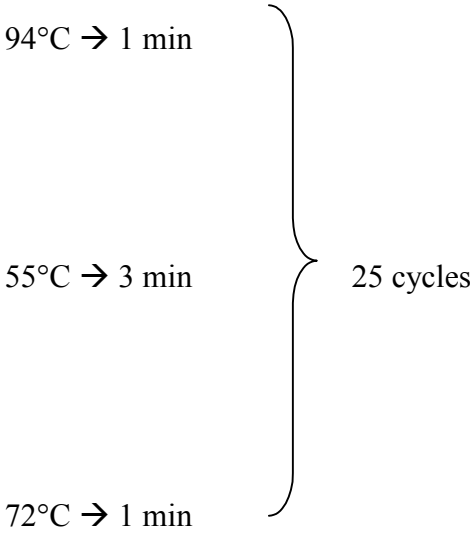
□□ Glycerol banks of cells were prepared as %85/ %15 in liquid culture/glycerol ratio.

□□ Competent cells used for transformation were prepared according to Sambrook J, 2001.

3.2.2 PCR (Polymerase Chain Reaction)

PBS-AGG1 vector taken from Dr. Botella was used for all PCR protocols. Annealing temperatures of primers with and without restriction sites were estimated according to the formula in the PCR Core System II (Promega). The following PCR conditions were followed in all amplification processes:

94°C → 1 min



72°C → 5 min

4°C → Hold

3.2.3 Purification of PCR product

PCR product was purified either from the 1% Agarose gel with Quiaquick® Gel extraction Kit (250) (QIAGEN) or directly with Quiaquick® PCR Purification Kit (250) (QIAGEN).

3.2.4 Agarose Gel Electrophoresis

□□ Plasmid isolation, restriction digestion analysis of samples on 1% Agarose at 105mV constant voltage for 50 min. Samples to be extracted from gel were run on 1.5% Agarose at 105mV constant voltage for 50 min.

3.2.5 SDS-Polyacrylamide Gel Electrophoresis

□□ Proteins to be observed on polyacrylamide were mixed with 5X loading buffer and 5% 2-Mercaptoethanol and denaturated at 95°C for 2.5 min. Polyacrylamide gels were prepared in the following ratios:

5% Stacking Gel	15% Seperating Gel
50 mM Tris-HCl pH 6.8	375 mM Tris-HCl pH 8.9
5% Acryl-bisacryl (30%-0.8%)	15% Acryl-bisacryl (30%-0.8%)
0.04% 20 % SDS	0.1% 20 % SDS
0.075% 10 % APS	0.075% 10 % APS
0.05% TEMED	0.05% TEMED

The gels were stained with Comassie staining.

3.2.6 Plasmid Isolation

□□ Purified plasmids containing were digested with appropriate restriction enzymes according suppliers instructions to verify the presence of GPA1. (Enzyme/reaction mix) v/v ratio was kept at 1/10 or smaller in all digestions.

3.2.7 Restriction Endonuclease Digestion

□□ Purified plasmids containing insert were digested with appropriate restriction enzymes according suppliers instructions to verify the presence of GPA1. (Enzyme/reaction mix) v/v ratio was kept at 1/10 or smaller in all digestions.

3.2.8 Ligation

□□ Isolated pGEX4T-2 was ligated with *AGG1* containing the same restriction site with the vector, with different insert:vector ratios. PCR amplified *AGG1* with restriction sites was ligated to pUC18 digested with those restriction sites, and directly to pGEMTEasy vector. PCR amplified *AGG1* with no restriciton sites was ligated directly to TOPO-T7, TOPO pTrcHis expression vectors and pCR II- TOPO subcloning vector. The reaction mixtures were incubated 5 min. at room temperature.

3.2.9 Transformation

□□ Ligation mixtures were transferred into different endonuclease deficient strains of *E. coli*- XL1 Blue, TOP10, BL21 (DE3) and BL21 (DE3) pLysS. Transformed cells were plated on appropriate antibiotic selective LB plates prepared according to the ligation vectors.

3.2.10 Colony Selection

□□ Positive colonies were selected and grown on liquid LB culture containing appropriate antibiotic selection for both preparing glycerol stocks and plasmid isolation.

3.2.11 Sequence Verification

□□ QIAGEN® Plasmid Midi Kit (100) (QIAGEN) purified plasmids containing *AGG1* were sent for sequence analysis.

3.2.12 Procedures used in subcloning

3.2.12.1 Subcloning of *AGG1* using pUC18

AGG1 was PCR amplified from AGG1-pBS plasmid using SacI restriction site in the forward primer and EcoRI restriction site in the reverse primer. Commercial pUC18 vector was digested in the following conditions:

	Volume (μL)
10X Tango Buffer	2
pUC18	5
HincII	1
ddWater	12
Total	20

□□ PCR purified *AGG1* was subjected to fill-in reaction using DNA polymerase Klenow fragment and digested pUC18 was dephosphorylated. These two samples, without extracting from the gel, were directly ligated in 10:1 insert:vector ratio (v/v). Tranformation protocol into TOP10 competent cells was followed according to Maniatis *et. al.* and 10 colonies were picked that 2 of them were verified by restriction digestion to contain the insert, one is in forward; the other is in reverse direction.

3.2.12.2 Subcloning of *AGG1* using pGEMT-Easy

□□ PCR amplified *AGG1* having SacI and EcoRI restriction sites was ligated with pGEMTEasy vector in a 3:1 insert:vector ratio. Transformation of TOP10 competent cells was followed by verification of the presence of insert by restriction endonuclease digestion.

3.2.13 Procedures used in expression cloning

3.2.13.1 Cloning of *AGG1* using TOPO-T7 expression vector

□□ PCR amplified *AGG1* is ligated with TOPO-T7 expression vector and transferred into *E. coli* BL21 (DE3) pLysS competent cells. The presence and direction of insert is verified by PCR and polyacrylamide gel electrophoresis.

3.2.13.2 Cloning of *AGG1* using TOPO-pTrcHis expression vector

□□ PCR amplified *AGG1* is ligated with TOPO-pTrcHis expression vector and transferred into *E. coli* BL21 (DE3) pLysS competent cells. The presence and direction of insert is verified by PCR and polyacrylamide gel electrophoresis.

3.2.13.3 Cloning of *AGG1* using pGEX4T-2 expression vector

□□ Both TOPO-AGG1 and pGEX4T-2 were digested with EcoRI and XhoI in the following conditions.

□□	Volume (μL)
DNA	5
EcoRI	1
XhoI	1
10X Tango Buffer	4
ddWater	9
Total	20

□□ The digested vectors were ligated in 1:1 insert vector ratio (w/w) and transferred into *E. coli* BL21(DE3) competent cells and the presence of insert was verified by restriction digestion.

3.2.14 Expression

□□ The expression cells containing *AGG1*+vector were subcultured with 5 ml LB+ampicillin and the day after diluted to 0.02 OD. Cultures were induced with 0.8 mM IPTG near O.D=1 and samples were taken at 2 hour intervals for 8 hours. The pellets were lysed, sonicated and run on 5-15% SDS-polyacrylamide gel.

3.2.15 Western Blotting

□□ 5-20% SDS-polyacrylamide gel was blotted on PVDF membrane at 25 mAconstant current for 3 hours. Blotted membrane was stained with Poncaeu S stain fro 15 min and washed with PBS. PVDF memnbrane was blocked with blocking solution containing 0.2% Tween and 5% non-fat milk powder, incubated with anti-His antibody for 2 hours and transferred to secondary antibody (HSP) solution where it was incubated for 2 hours. The membrane was applied with Pharmacia Chemiluminescent detection solution and resulting bands were identified using Pharmacia ECL+ X-ray film.

4 RESULTS

4.1 pBS-AGG1 and pBS-AGG2 plasmids

□□ The AGG1-pBS plasmid was kindly provided by Dr. Botella as blotted on paper. DNA was transferred into solution by cutting the blotted spot and with vigorous shaking in TE buffer. As can be seen in figure 4.1, AGG1-pBS and AGG2-pBS were visualized on 1% Agarose gel before further manipulation:

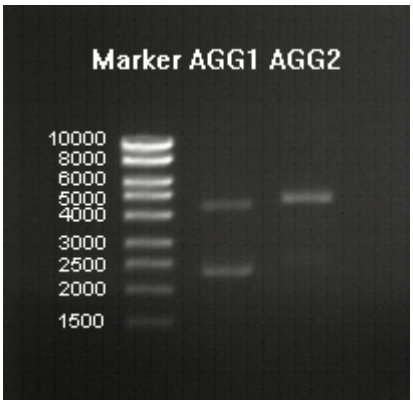


Figure 4.1 Agarose gel electrophoresis of AGG1-pBS and AGG2-pBS plasmids

□□ According to the plasmid map supplied by Dr. Botella, *AGG1* fragment had been inserted between Kpn I and Spe I restricton enzyme sites and could be excised by these enzymes to appear as a ~ 420-base fragment , as shown in figure 4.2 on 1% Agarose

gel. A 297 bp region of this fragment was expected to correspond to *AGG1*. The actual verification of *AGG1* was carried out by sequencing. A 700 bp sequenced region of AGG1-pBS vector is given in Appendix C and *AGG1* sequence is between nucleotides 71 and 367 in this sequence.

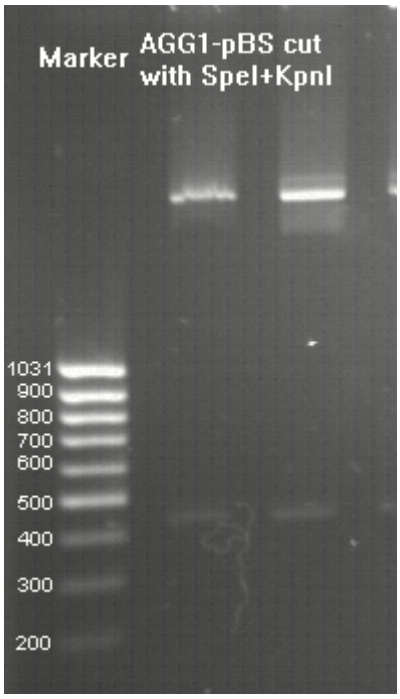


Figure 4.2 Agarose gel electrophoresis verifying the presence of *AGG1* in AGG1-pBS

4.2 Subcloning and Sequence verification of *AGG1*

4.2.1 Insertion into pUC18

4.2.1.1 PCR amplification of *AGG1*

AGG1 was amplified using the following primers. Sac I restriction site is present in Forward primer and EcoRI restriction site is present in Reverse primer. These restriction sites are underlined.

Forward primer: 5'- TATATAGAGCTCATGCGAGAGGAAA- 3' and

Reverse primer: 5' – GCGGCGGAATTCTCAAAGTATTAAG- 3'

A 6 bp additional sequence was used in primers before the restriction sites. This additional sequence is different in the forward and reverse primers, to equalize the annealing temperatures, making use of different bond strengths of A-T and G-C pairs.

All PCR amplifications, both for cloning and subcloning were performed using the AGG1-pBS vector as template. PCR amplification using the above primers were used for also subcloning into pGEMTEasy vector. Results of amplification of *AGG1* were monitored by agarose gel electrophoresis. As can be seen on figure 4.3 a fragment of about 300 bp length was obtained as the PCR product

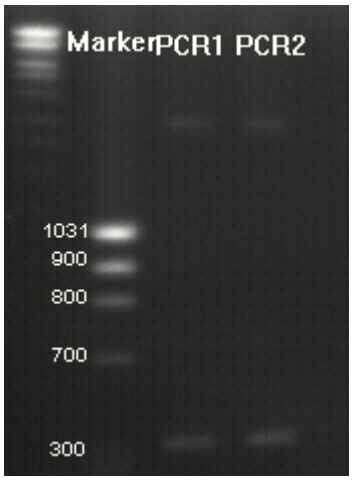


Figure 4.3 PCR amplification of *AGG1*

4.2.1.2 Ligation into pUC18

□□ To prepare a pUC18-AGG1 construct for using as a subcloning vector in order to make a GFP-AGG1 fusion protein, the amplified *AGG1* was tried to be ligated into pUC18. PCR amplified *AGG1* was gel purified and the flanking regions were filled with DNA Polymerase Klenow fragment, resulting in blunt ends. The commercial pUC18 was also cut with the same enzyme and the two mixtures were ligated directly, without gel purification. *E. coli* TOP10 cells were transformed with potential construct and 10 colonies were selected according to blue-white colony screening using ampicillin resistance as selective agent. Plasmids isolated from liquid cultures of these cells are shown in figure 4.4. pUC18 plasmid normally have SacI and EcoRI Restriction sites in its MCS. Digestion of the possible construct using SacI will result in a band of ~325 bp if it is inserted in the forward direction, the same band will be observed using EcoRI if it is inserted in the reverse direction. Restriction digestion of these plasmids using SacI and EcoRI, separately, resulted in 2 inserts: one in the forward direction (named A1) and one in the reverse direction (named A9), on 1% Agarose gel, which is shown in figure 4.5. Subsequently clones A1 and A9 were sequenced verifying the presence of *AGG1* in the pUC18 vector.

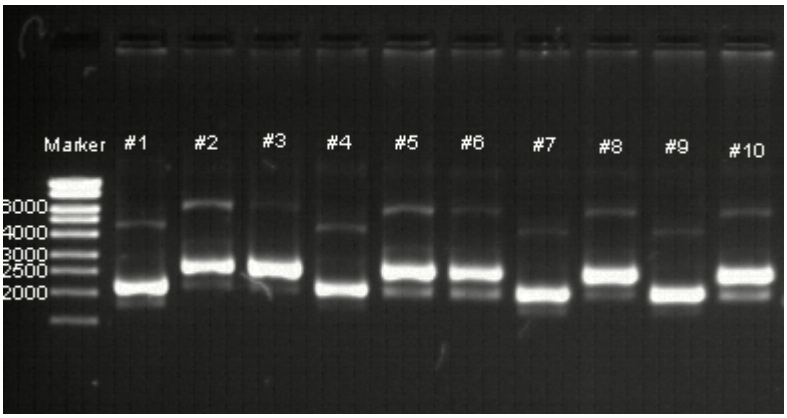


Figure 4.4 Isolated possible constructs

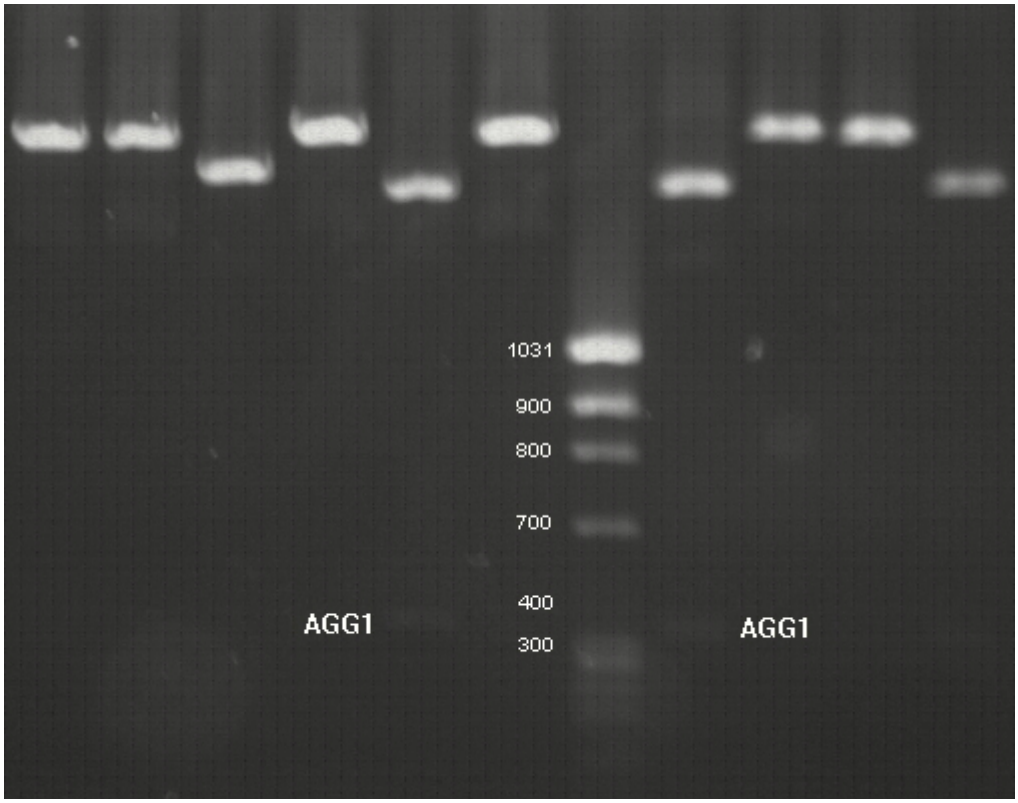


Figure 4.5 Verification of *AGG1* in pUC18 by Agarose gel electrophoresis using SacI and EcoRI restriction enzymes.

4.2.2 Insertion into pGEMTEasy

□□ PCR amplified *AGG1* was gel purified, inserted into pGEM T-Easy vector and TOP10 cells were transformed with the construct. 10 *AGG1* positive colonies were

selected according to blue-white colony screening using ampicillin resistance. Plasmids were isolated from these cells in liquid cultures. . pGEM T-Easy vector facilitates A-tailing ligation of the fragment between two flanking Eco RI sites and presence of the insert can be verified by digestion of the isolated plasmids with Eco RI. The restriction digestion result on 1% Agarose gel is shown in figure 4.6. One plasmid (named B2) was verified by sequencing.

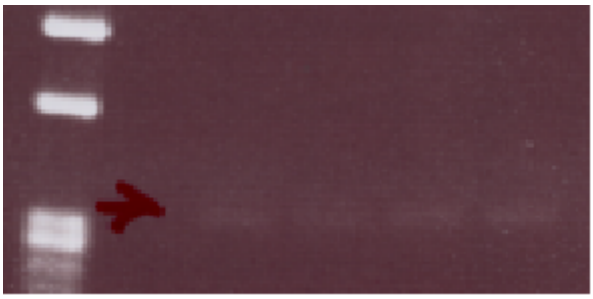


Figure 4.6 Verification of *AGG1* in pGEMTEasy by Agarose gel electrophoresis using SacI and EcoRI restriction enzymes. Lane 1: Marker bands corresponding to 500, 400, 300, 200, 150 bps respectively. Lanes 3-6: *AGG1*

4.2.3 Insertion into pCRII-TOPO

□□ AGG1+PCR-TOPOII construct was prepared as a first step in constructing AGG1-pGEX4T-2 vector. Hence, primers were designed in order to insert *AGG1* in-frame into pGEX4T-2. *AGG1* was amplified from AGG1-pBS using the following primers. The forward primer contains EcoRI Restriction site while the reverse primer contains XhoI Restriction site. These Restriction sites are underlined below:

Forward primer: 5'- GAATTCCATGCGAGAGGAAACTGTGGTTTAC- 3' and

Reverse primer: 5' – CTCGAGTCAAAGTATTAAGCATCTGCAGCC- 3'

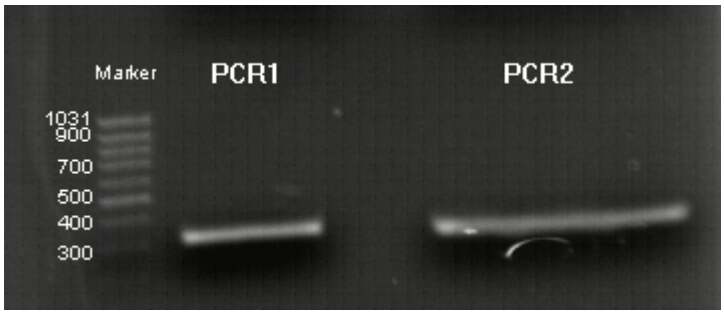


Figure 4.7 PCR amplification of *AGG1* from AGG1-pBS fro insertion into pCRII-TOPO plasmid.

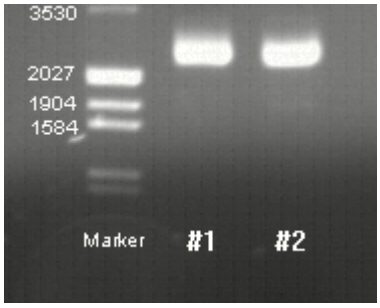


Figure 4.8 Plasmid isolation of possible AGG1-pCRII-TOPO constructs.

□□ Gel purified PCR product was directly ligated with PCR-TOPO subcloning vector and transferred into TOP10F' competent cells. 2 light blue colonies were picked and one of them was verified to contain *AGG1* by restriction digestion using EcoRI + XhoI, on 1% Agarose gel.

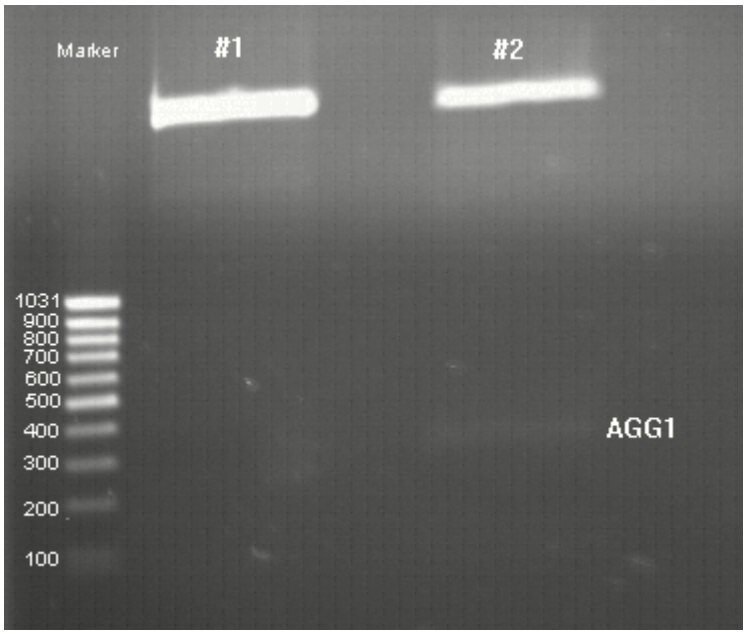


Figure 4.9 Restriction digestion using EcoRI+XhoI. One of the possible constructs did not contain *AGG1* (#1) while the other did (#2).

4.3 Cloning of *AGG1* using expression vectors

4.3.1 Insertion into TOPO-NT7 and TOPO-pTrcHis

TOPO-NT7 and TOPO-pTrcHis are two commercial expression vectors (Invitrogen) for directly inserting PCR products. As shown in the vector maps in Appendix A, ligation is achieved by making use of two Adenine bases added during PCR, complemented with the action of Topoisomerase. Since subcloning procedure is bypassed and the gene is directly inserted into the expression vectors. Primers containing no restriction sites were designed and used. *AGG1* was PCR amplified using the below primers. Two same samples were observed preparatively on 1% Agarose gel, which were then added to each other, for getting rid of overloading.

Forward primer: 5'- ATGCGAGAGGAAACTGTGGTTTAC- 3' and
Reverse primer: 5' – TCAAAGTATTAAGCATCTGCAGCC- 3'

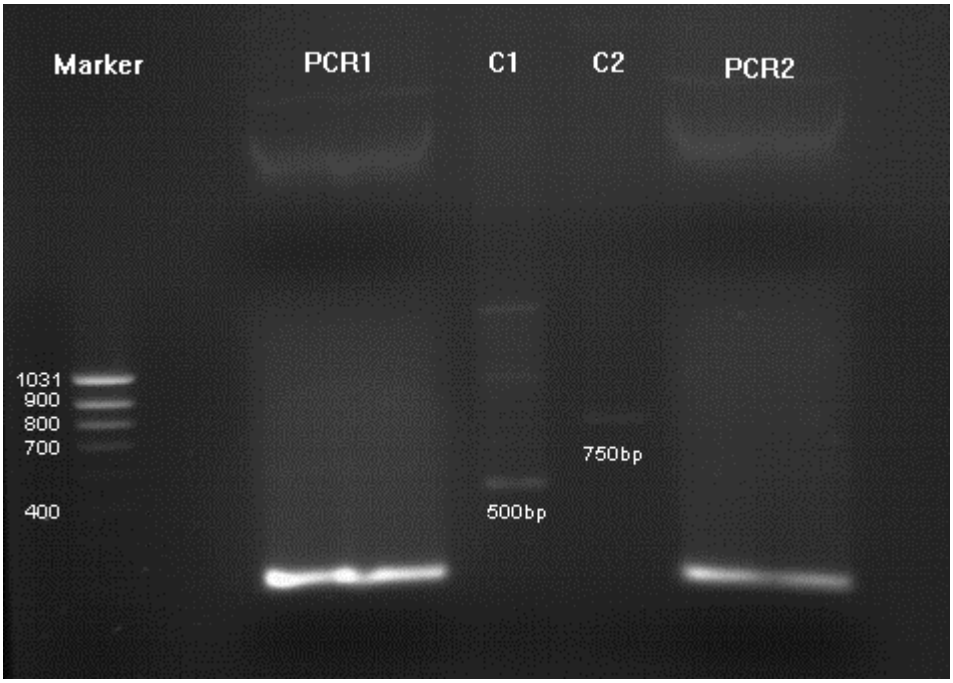


Figure 4.10 PCR amplification of *AGG1* from AGG1 pBS plasmid for insertion into TOPO expression vectors. Lanes 3 and 4 (C1 and C2) are control reactions of PCR.

□□ Gel purified *AGG1* was directly ligated with different amounts of TOPO-NT7 and TOPO-pTrcHis expression vectors. TOP10F' competent cells were transformed with potential constructs and presence and direction of inserts were checked by restriction endonuclease digestion. The vectors contain useful Restriction sites at sites of insertion, as shown in Appendix A. For NT7, the presence of insert was verified by using NdeI + HindIII. For pTrcHis, the presence of insert was verified by using BamHI+EcoRI.

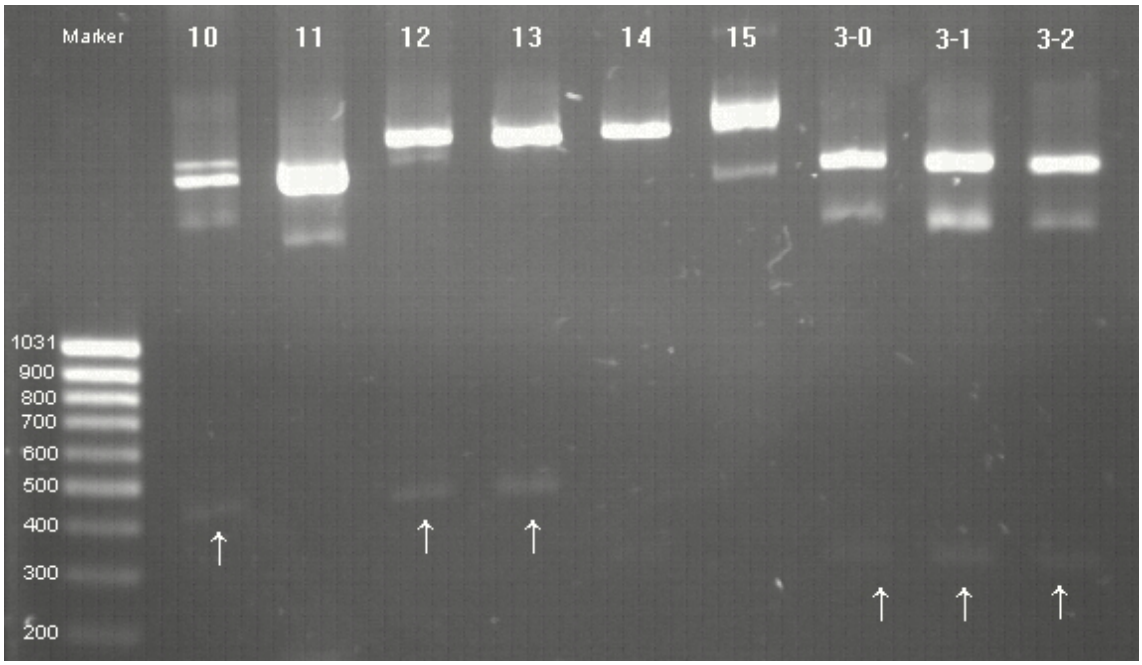


Figure 4.11 Agarose gel electrophoresis verifying the presence of *AGG1* in TOPO expression vectors. Lanes 2-7 (10-15) include isolated possible AGG1-TOPONT7 constructs digested with NdeI+HindIII. Lanes 8-10 (3-0, 3-1, 3-2) include isolated possible AGG1-TOPOpTrcHis constructs digested with BamHI+EcoRI.

□□ The verification of insertion direction was done by PCR. A combination of Forward primer used for PCR amplification of *AGG1* and Reverse primer supplemented with the vector kits were used. With this combination, if a band around 300 bp is observed, the direction would be correct. *AGG1* forward primer and TOPO reverse primer was used and the direction of the inserts were determined. 1% Agarose gel Electrophoresis is showing that insertion is achieved in the correct direction.

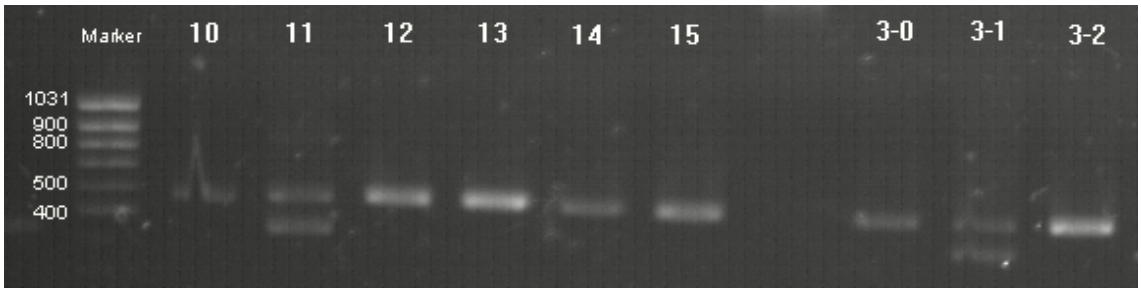


Figure 4.12 Verification of the direction of inserts by PCR using *AGG1* forward primer and TOPO reverse primer. Result shows that all are insert are in forward direction.

4.3.2 Insertion into pGEX4T-2

□□ AGG1-TOPO construct was digested with EcoRI and XhoI restriction enzymes, and extracted from 1.5% Agarose gel. PGEX4T-2 plasmid, isolated from *E. coli* BL21 series was also digested with the same same ezymes and ligated with *AGG1*. The construct was transferred into both XL1Blue and BL21 competent cells. The presence of the insert was verified by restriction digestion, observed on 1% Agarose gel.

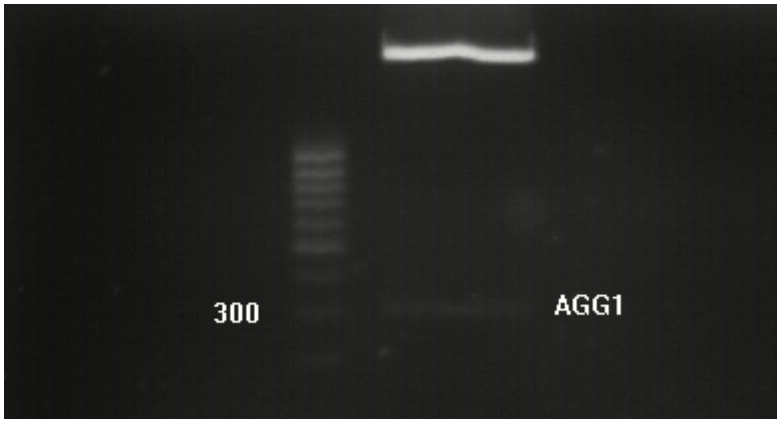


Figure 4.13 Agarose gel elctrophoresis showing the presence of *AGG1* in pGEX4T-2 plasmid.(Lane1: Fermentas Mass Ruler Low Range Molecular Weight Marker)

4.4 Expression

4.4.1 Induction of BL21 (DE3) cells containing TOPO-NT7+ AGG1

□□ *E. coli* BL21 (DE3) cells carrying TOPONT7-AGG1 were induced using 0.8mM and 1.25mM IPTG for 8 hours and appropriate amounts of samples were taken at 2-hour intervals. 10 uL samples were loaded on 5-15% SDS-polyacrylamide gel. As seen from the gel photos, no expression of *AGG1* were observed, when compared with the empty BL21 (named B) and non-induced cells (named -).

	Initial	t=0	t=1 (2 h.)	t=2 (4 h.)	t=3 (6 h.)	t=4 (8 h.)	t=5 (o/n)
BL21 DE3	0.22	0.75	2.58	3.4	3.9	4.19	10
13 (-)	0.3	0.93	2	3.21	3.01	3.3	2.7
13 (+) (0.8mM)	0.31	1.04	2.35	2.78	2.98	3.16	9.42
13 (+) (1.25mM)	0.31	1.04	2.34	2.76	2.79	2.9	2.11

Table 4.1 OD measurements of cultures taken at 2 hour intervals. (+) cells are induced at t=0

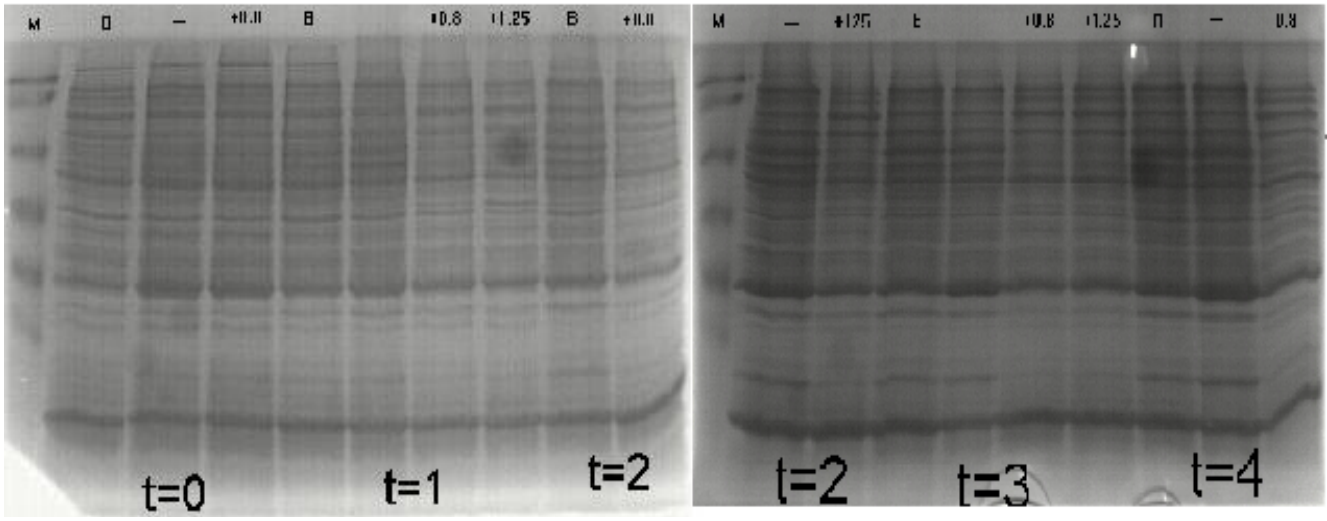


Figure 4.14 SDS-polyacrylamide gel electrophoresis of samples taken from cells at above specified time points. (Solubilized samples)

Lane #	Sample	Lane #	sample
1	Molecular Weght Marker	6	BL21 (DE3) at t=1
2	BL21 (DE3) at t=0	7	13 (+0.8mM IPTG) at t=1
3	13 (-) at t=0	8	13 (+1.25mM IPTG) at t=1
4	13 (+) at t=0	9	BL21 (DE3) at t=2
5	BL21 (DE3) at t=1	10	13 (+0.8mM IPTG) at t=2

11	Molecular Weght Marker	16	13 (+0.8mM IPTG) at t=3
12	13 (-) at t=2	17	13 (+1.25mM IPTG) at t=3
13	13 (+1.25mM IPTG) at t=2	18	BL21 (DE3) at t=4
14	BL21 (DE3) at t=3	19	13 (-) at t=4
15	13 (-) at t=3	20	13 (+0.8mM IPTG) at t=4

Table 4.2 Sample description for Figure 4.13

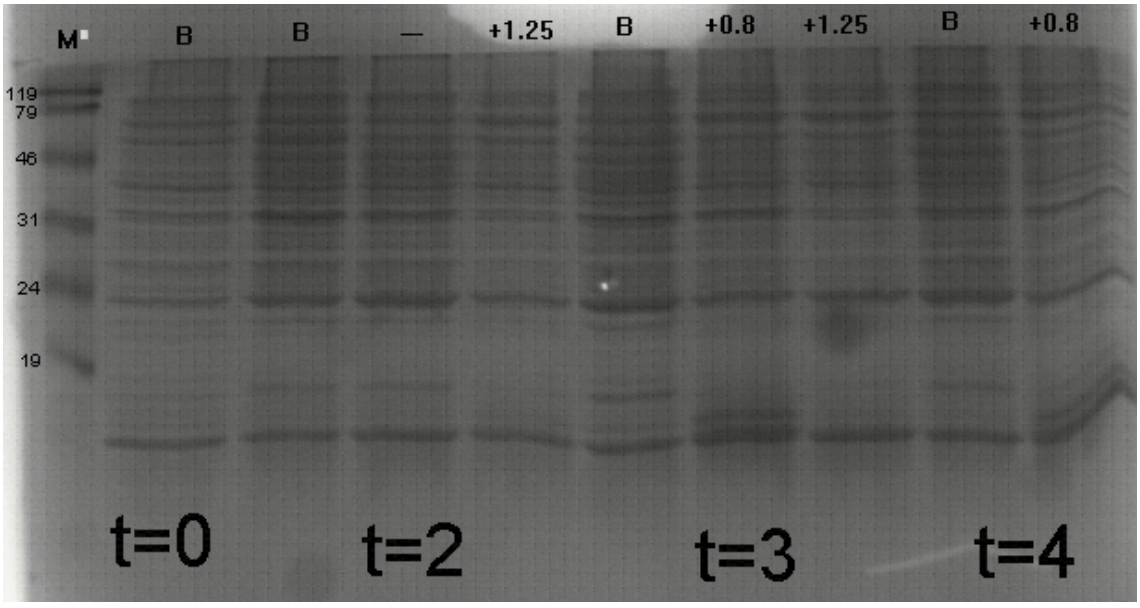


Figure 4.15 SDS-polyacrylamide gel electrophoresis of samples taken from cells at above specified time points. (pellet samples). Lane 2: t=0, Lanes 3-5: t=2, Lanes 6-8: t=3, Lanes 9-10: t=4

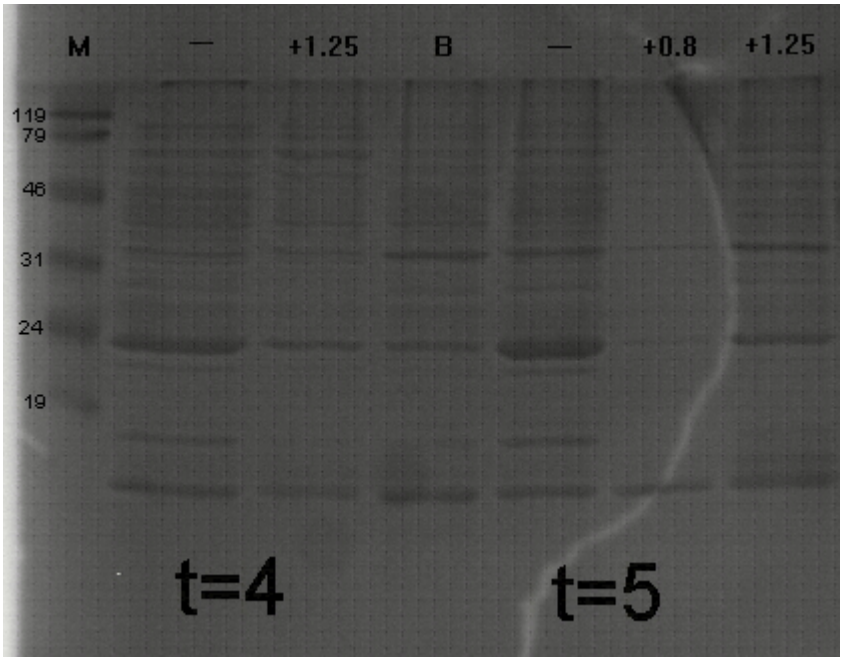


Figure 4.14 (continued): Lanes 2-3: t=4, Lanes 4-7: t=5

4.4.2 Induction of BL21 cells containing TOPO-pTrcHis+AGG1 construct

□□ *E. coli* BL21 (DE3) cells carrying TOPOpTrcHis-AGG1 were induced with 0.8mM IPTG for 6 hours and appropriate amounts of samples were taken at 2-hour intervals.

	Initial	t=0	t=1 (2 h.)	t=2 (4 h.)	t=3 (6 h.)
BL21 DE3		0.25	1.16	2.31	2.84	3.18
Control		0.8	0.91	2.17	2.74	3.27
3-2 (-)		0.12	0.68	1.73	2.36	2.17
3-2 (+)		0.08	0.72	1.03	0.81	0.284

Table 4.3 OD measurements of cultures taken at 2 hour intervals. (+) cells are induced at t=0

□□ 10 µL samples were loaded on 5-15% SDS-polyacrylamide gel. As seen from the gel photos, no expression of *AGG1* were observed, when compared with the empty BL21 (labeled B) and non-induced cells (labelled -). C denotes pTrcHis expression control.

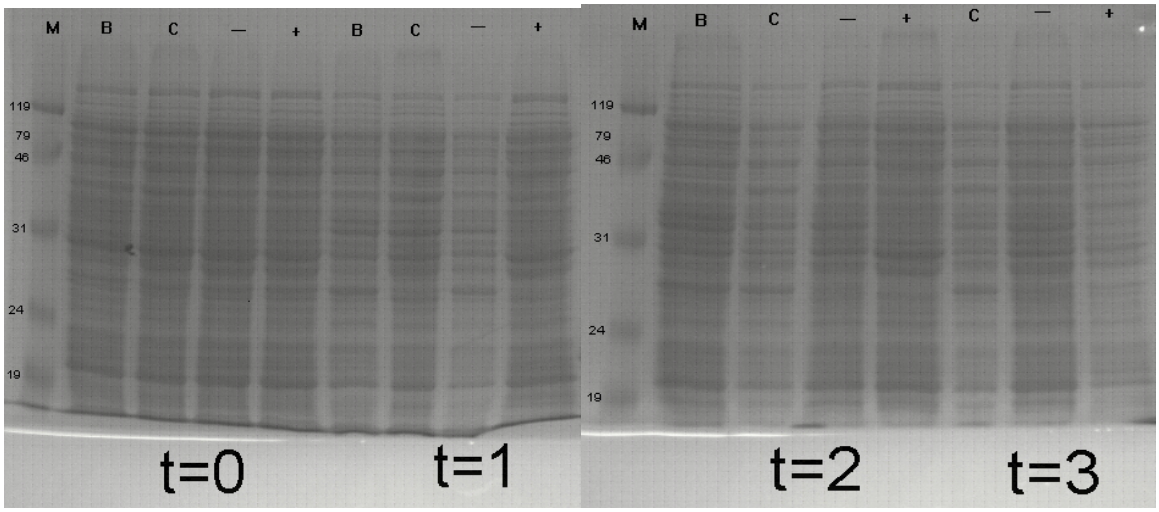


Figure 4.16 SDS-polyacrylamide gel electrophoresis of samples taken from cells at above specified time points. Lane 1: Molecular Weight Marker (Fermentas), Lanes 2-5: t=0, Lanes 6-9: t=1, Lane10: Molecular Weight Marker (Fermentas), Lanes 12-15: t=2, Lanes 16-19: t=3

Large scale (200 ml.) *E. coli* BL21 (DE3) cells carrying TOPOpTrcHis-AGG1 were induced with 1 mM IPTG for 4 hours. According to the assumption that the protein is toxic to *E. coli* strain, cells are induced at a relatively high concentration (near O.D=1.5) and samples were taken at smaller time intervals. Sampling has continued until possible toxicity has decreased cell concentraion (O.D= 1.93).

	Initial	t=0	t=1 (30min)	t=2 (60min)	t=3 (120min)	t=4 (240min)	
BL21 DE3		1.13	2.36	2.89	3.54	3.93	5.7
3-2 (-)		0.63	1.77	2.23	2.71	2.82	5.11
3-2 (+)		0.68	1.66	1.68	1.99	2.39	1.93

Table 4.4 O.D. measurements taken at specified time points. (+) cells are induced at t=0

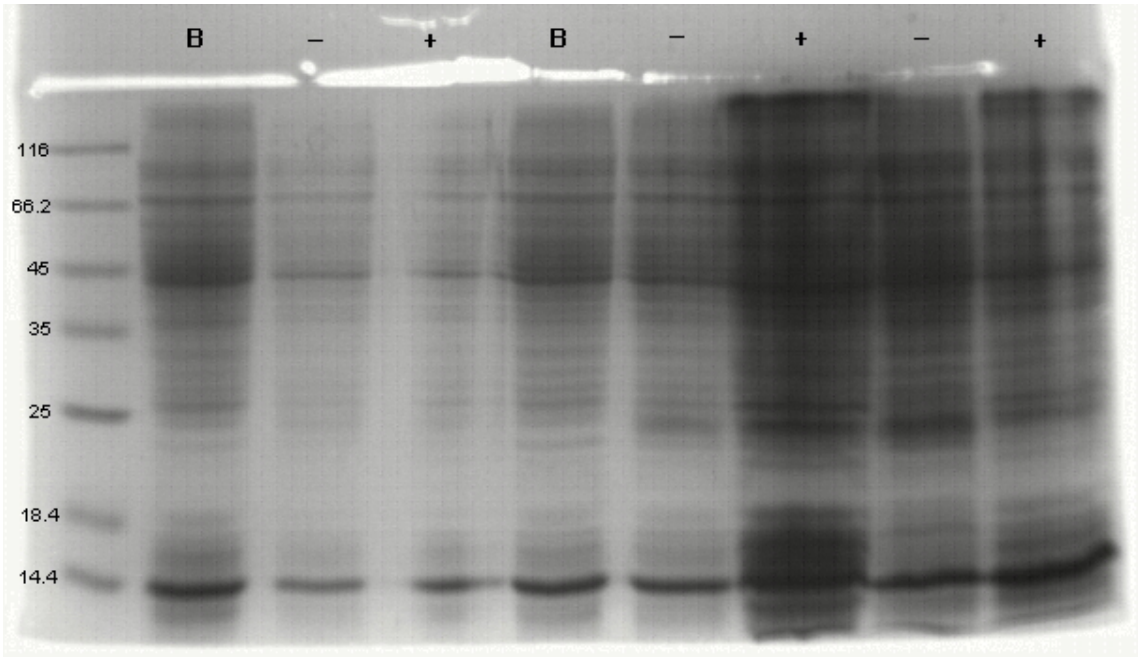


Figure 4.17 SDS-polyacrylamide gel electrophoresis of samples taken from cells at above specified time points

Lane #	sample	Lane #	sample
1	Molecular Weght Marker	6	3-2 (-) at t=2
2	BL21 (DE3) at t=0	7	3-2 (+) at t=2
3	3-2 (-) at t=0	8	3-2 (-) at t=4
4	3-2 (+) at t=0	9	3-2 (+) at t=4
5	BL21 (DE3) at t=2	10	

Table 4.5 Sample description of Figure 4.16

The induced cultures were speculated to synhesize maximum number of protein at t=2, so t=2 sample was used for Western blot analysis. For the verification of correct localization of bands on PVDF membrane, the SDS gel was loaded with the samples twice. One half was comassie stained and the other half was blotted onto membrane. In addition, a second protein known to contain His-tag was used as the control of the study. The localization of the two bands on membrane matches correctly to the ones on SDS gel as shown in Figure 4.18, hence the two bands on the membrane are pETM_GFP_Imm (control) and AGG1, respectively.

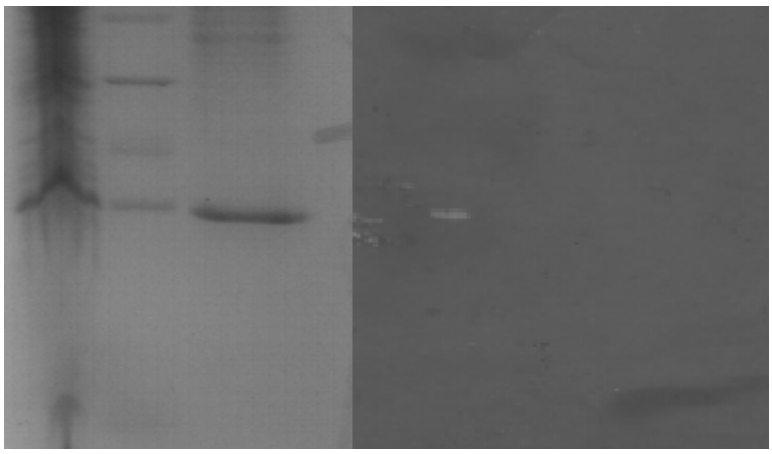
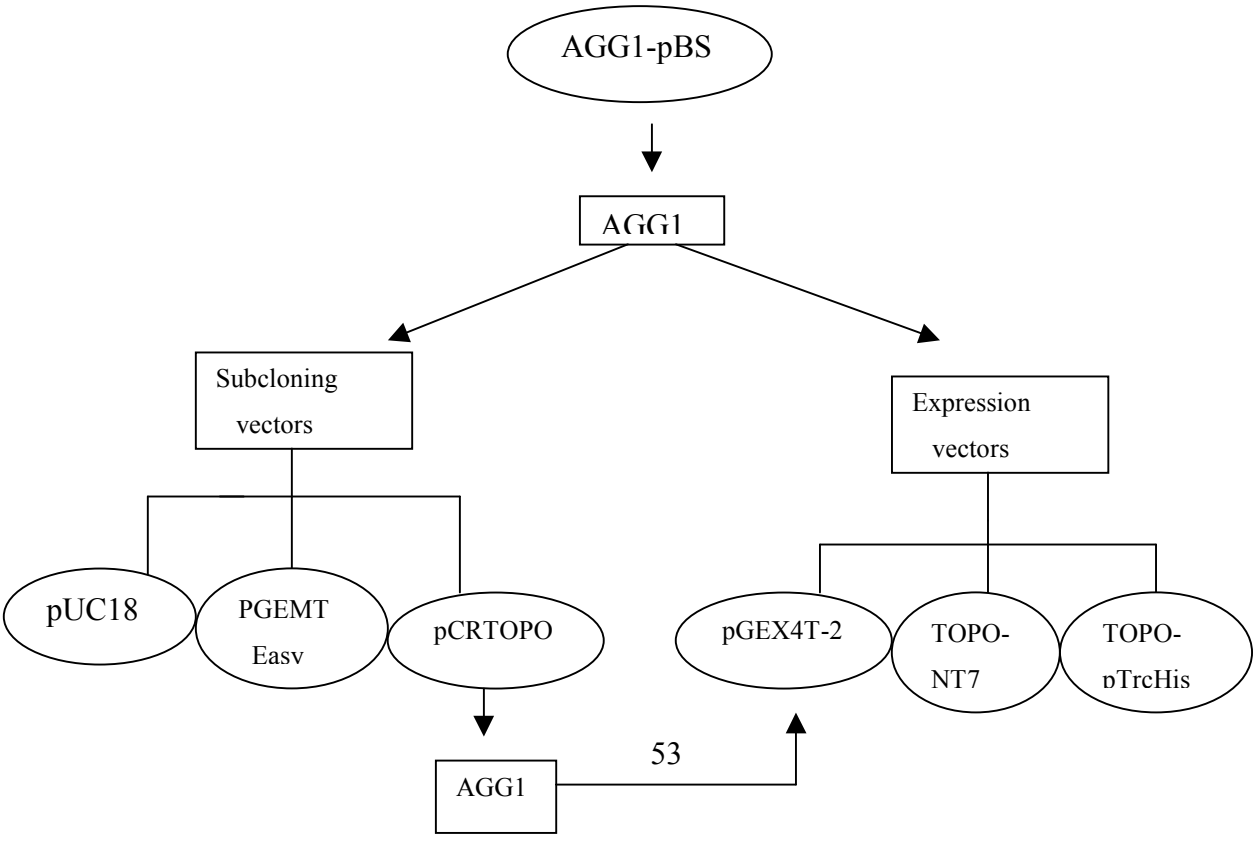


Figure 4.18 5-20% SDS-PAGE comassie stained and correspondent Western Blot analysis of samples taken from induced cells at t=2. Left: Lane 1: t=2 induced cells, Lane 2: Protein MW marker (bands corresponding to 35, 25, 18.4, 14.4 kDa from top to bottom) Lane 3: pETM_GFP_Imm protein containing His tag. Right: Western Blot of left gel. Lane 1: pETM_GFP_Imm, Lane 3: AGG1

5. DISCUSSION

□□ The main and long-term aim of the study is to solve the structure of plant G protein γ-subunit using X-ray crystallography and study the interactions of the subunits in the plant heterotrimer (if such a functional entity exists) using small angle X-ray scattering. This requires the cloning and high-yield expression of the gene in an optimal host strain and efficient purification of the resulting protein. *AGG1* gene, provided initially in pBS vector has been inserted into 3 subcloning and 3 expression vectors and 3 of them (AGG1-pUC18, AGG1-pGEMTEasy, AGG1-TOPOpTrcHis) have been shown to be cloned in frame and without mutation. Expression of AGG1 protein in *E. Coli* host strains has been demonstrated by western blot analysis.



□□

□□ Working with AGG1 has had special challenges due to its low molecular weight protein, only 7 kD, it existing in a tight complex with G-beta in ammalian and other systems where it has been identified and due to possible complications in stablising the 3D fold of this small protein in an prokaryotic system.

5.1 Cloning

AGG1-pBS plasmid, which was kindly provided by Dr. Botella (University of Quennsland, Australia) was firstly sequenced to verify the location and the sequence of the gene. This proved to be justified since the other construct named as AGG2-pBS by Dr. Botella was shown not to contain *AGG2* gene correctly after two sequencing attempts carried out by different sequencing companies.

□□ During early stages of the project attempts were made to insert *AGG1* gene into pUC18 subcloning vector and then to transfer it to the pGFPuv vector for expression as a fusion protein together with GFP. For this purpose, a forward primer containing SacI restriction site and a reverse primer containing EcoRI restriction site were designed. These would allow cloning of *AGG1* in the 3' MCS (Multiple Cloning Site) of pGFPuv, excising –hence bypassing- the stop codon of GFP gene, without interfering with the chromophore region. The stop codon of *AGG1* would therefore be used as the stop codon of GFP-AGG1 fusion protein. However, several attempts failed to produce the pGFPuv+AGG1 construct (data not shown in results section). As can be seen from the vector map of pGFPuv in Appendix A, SacI and EcoRI restriction sites are very close to each other (~10 bases). This can inhibit the correct fitting of two different enzymes to their targets because of competing for nearly the same region on DNA. The inhibition of binding probably resulted in failure of expected sticky ends because the excision of

AGG1 from pUC18 vector using the same restriction enzymes was succesful in every trial.

The subcloning of *AGG1* into pUC18 was carried out in an unusual way. Instead of gel extraction of digested vector followed by ligation, the HincII cut pUC18 was left in tube and ligated with PCR amplified *AGG1* subjected to fill-in reaction directly. This resulted in a combination of false positive transformants containing self-ligated pUC18 and as well as those containing AGG1-pUC18 construct, verified by restriction digestion. A blunt-end ligation, which failed several times in other studies, was achieved.

pCRII-TOPO vector making use of the topoisomerase enzyme was used for subcloning. The resulting construct was digested with EcoRI and and XhoI, which were the sites coming from previously designed primers in order to insert *AGG1* into pGEX4T-2 plasmid. The AGG1-pGEX4T2 construct was succesfully prepared but sequencing result showed an A to C single base mutation at the start codon of *AGG1*, which can be due to either wrong synthesis (not design) of primer, wrong base pairing in PCR or UV light exposure during the extraction from Agarose gel for purification.

5.2 Expression

□□ Construction of fusion proteins is a convenient way of expressing genes in organisms other than their natural origins. Every organism beginning from bacteria to the ones having large genomes developed defense mechanisms to -unwanted- molecules. Protection for the recombinant protein can be achieved using a fusion partner for the gene of interest, especially for the small ones, to lessen the effect of the defense mechanisms inside the host. Among these partners GFP would be a good choice with its stable 3-D structure coming from a central chromophore region covered with a beta-barrel composed of eleven beta strands at the sides and distorted alpha helices at both ends (Philips, G.N.Jr., 1997). GST, a ~30 kDa less stable protein, is another

candidate. In this study, GFP fusion could not be achieved and GST fusion protein failed to be expressed due to the above mentioned point mutation.

One of the cloned TOPO direct expression vectors was sequenced and did not contain *AGG1* gene correctly. The other, being pCRTOPO-pTrcHis, was verified by sequencing to contain *AGG1* in-frame. *E. coli* BL21 (DE3) pLysS strains were transformed with construct and induced with 0.8, 0.9, 1, 1.25 mM IPTG (only 0.9 and 1mM concentrations shown in results section). In all experiments, optical density of the construct carrying cells showed a relatively low increase compared to the empty cells and the cells carrying a control plasmid. In addition, the induced cultures usually did not grow when they were left overnight. These results suggest potential toxicity of *AGG1* in the host strains.

Based on the assumption that the gamma subunit is toxic to transformed strains, induction of cells at higher cell density has been tried. Any toxic protein will be fatal for the cell if it is allowed to accumulate up to a certain concentration. So, if the number of cells are incresead, it will possible to collect/detect the protein at low accumulated amount at an early stage of synthesis before it reaches the fatal concentration in each cell. Therefore, liquid cultures were grown until they reach O:D ~ 1.5 and then induced with IPTG while in other experiments induction is done normally at 0.5-1 O.D. Moreover, *E. coli* (DE3)pLysS strains were selected for expressing a comperatively lower amount of T7 RNA polymerase for expression of toxic genes. pLysS does not effect the growth rate of host while increasing the stability of expression plasmid.

As mentioned at the beginning, there are particular difficulties in cloning G protein gamma subunit in bacterial systems compared to an ordinary cloning study. First, the protein is very small (about 7-10 kDa) and 3-D structure of its mammalian counterparts does not seem to be stable. As can be seen in figure 5.1, the two helical domains are free to rotate and very accessible to external effects.

This implies that the recombinant protein would be open to protease attacks in the host cell. It is known that bacterial systems contain mainly serine proteases and PMSF (phenyl- methyl sulfonyl fluoride) inhibits the activity of those proteases which was

used in all experiments. In the last trial of expression studies, a protein inhibitor cocktail purchased from Roche Chemicals (Complete) was used which blocks all serine and metalloproteases.

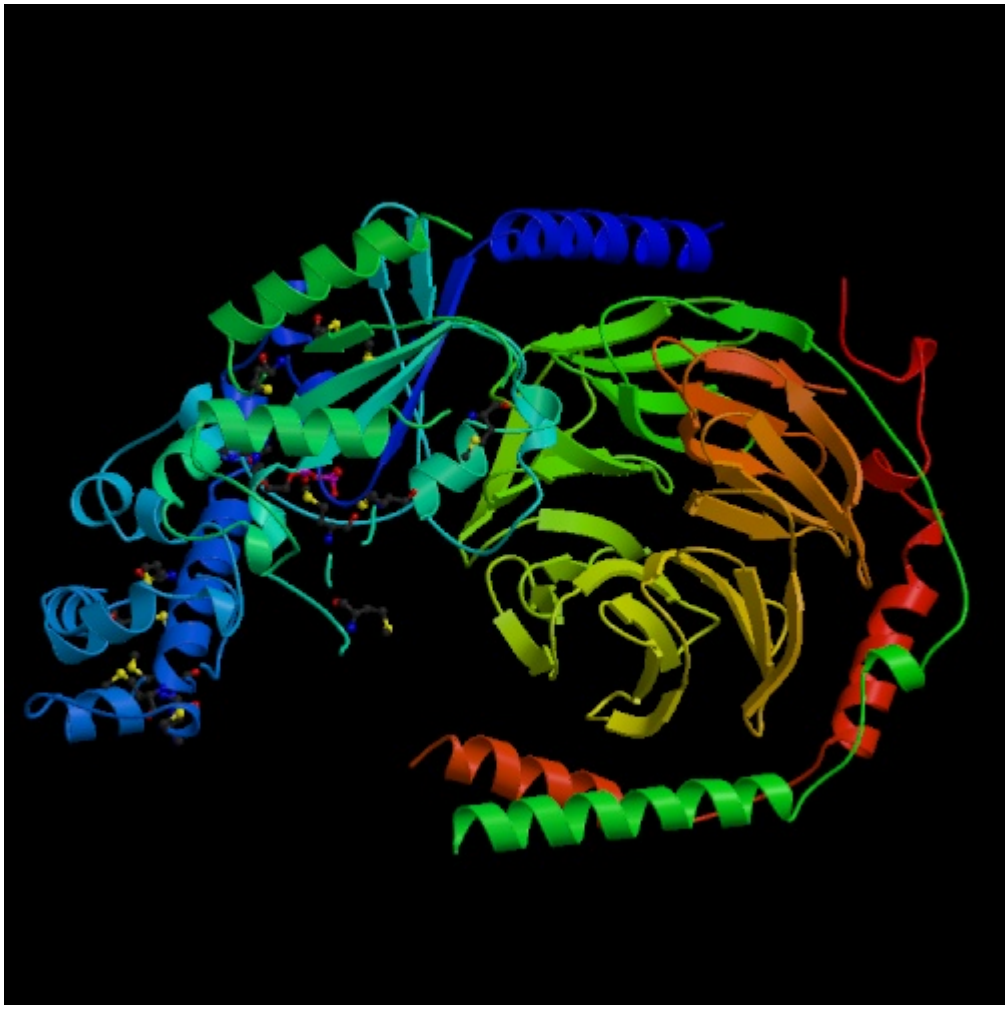


Figure 5.1 Heterotrimeric Complex Of A Gt-α/Gi-α Chimera and The Gt-β-γ Subunits. Gamma subunit is shown in red.

□□ Second, G protein gamma subunit is normally found in cells complexed with the beta subunit and it is impossible to separate the two subunits except for highly denaturing conditions. No intracellular effector molecule has been reported to be bound solely by the gamma subunit. Expression only of the gamma subunit gene will definitely change the usual folding pattern of the resulting protein which can effect level of synthesis. Because this is not a functional study, this is not that important but for X-ray experiments, deduced structure of the gamma subunit should be compared with the structure of beta-gamma dimmer.

Third, G protein gamma subunits found in eucaryotic organisms are subject to post-translational modifications occurring as lipid modifications which maintains both membrane anchoring and correct βγ binding. These modifications are absent in prokaryotes which might lead to folding problems resulting in degradation.

In spite of these difficulties concerning expressing such a protein having eucaryotic origin, in a prokaryotic system, a combination of a number of approaches resulted in the detection of protein synthesis. Large scale culture preparation, efficient inhibiton of proteases, induction at high cell concentration are some these approaches. In addition, *AGG1* is very difficult to detect by SDS-polyacrylamide gel electrophosis because it is very small. It could not be detected in several SDS-PAGE experiments possibly due to diffusing out of the gel. In the last trial, samples taken from induced cultures were run on a 5-20% denaturing gel and blotted on PVDF membrane. Western blotting resulted in the detection of His-tagged *AGG1*, which was verified by using another His-tagged protein as control.

Now that the G protein gamma subunit gene has been synthesized in a prokaryotic system, the optimization of recombinant protein in terms of stability and quantity should be performed. A more stable fusion protein involving AGG1 may be constructed trying GST and GFP fusions again. In addition, because the natural partner of AGG1 is the beta subunit, the dimer can be co-expressed by forming a beta-gamma construct. It is also known that GST gene is high highly expressed and GST protein is synthesized in high levels in the prokaryotic strains used. This can result in adequate quantity of AGG1 production for structural studies if an AGG1-GST fusion is constructed.

6. CONCLUSION

Cell behavior is regulated by a complex network of intracellular and extracellular signal transduction pathways. Guanine Nucleotide Binding Proteins (G proteins) are intermediate molecules in this network whose structure and function should be well characterized for understanding the whole mechanism.

Structural studies require high amount pure proteins. Both in academic studies and requirements in industry, deplete of natural resources lead to inevitability of genetic engineering, by production of useful proteins in host organisms having very short life spans. In this study *A. thaliana* G protein gamma subunit gene was cloned and expressed in prokaryotic systems, for future structural studies.

The selected organism was forced to express the gene and synthesize AGG1. However, due to possible toxicity of the gene and degradation of protein due to several reasons, synthesis level is low and may not be adequate for structural studies. Therefore, both construction of more stable fusion proteins of AGG1 should be carried out and expression studies should be done in other host organisms, including different strains of *E coli* and different prokaryotic systems as well as insect cells and yeast.

7. REFERENCE

1. Aharon G.S., Gelli A., Snedden W.A., 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 S.M., Jones A.M., Ullah H., Wang X-Q., " G protein regulation of ion channels and abscisic acid signaling in *Arabidopsis* guard cells" *Science* 292 (2001) 2070-2072

3. Baldwin J.M., "The probable arrangment of the helices in G protein-coupled receptors" *The EMBO Journal* 12 (1993) 1693-1703

4. Bockaert J., Pin J.P., "Molecular tinkering of G protein-coupled receptors: an evolutionary success " *The EMBO Journal* 18:7 (1999) 1723-1729.

5. Bohm A., Gaudet R., Sigler P.B., "Structural aspects of heterotrimeric G-protein signalling" *Current Opinion in Biotechnology* 8 (1997) 480-487

6. Botella J.R., Mason M.G., "Isolation of a novel G-protein γ-subunit from *Arabidopsis thaliana* and its interaction with Gβ" *Biochimica et Biophysica* 1520 (2001) 147-153.

7. Botella J.R., Mason M.G., “Completing the heterotrimer: Isolation and characterization of an *Arabidopsis thaliana* G-protein γ-subunit cDNA” *PNAS* 97:26 (2000) 14784-14788.

8. Bourne H.R., Wilson P.T., Wedegaertner P.B., “ Lipid modifications of trimeric G proteins” *The Journal of Biological Chemistry* 270:2 (1995) 503-506

9. Carter A.N., Downes C.P. “Phosphatidylinositol 3-kinase is activated by nerve growth factor and epidermal growth factor in PC12 cells” *J. Biol. Chem.* 267 (1992) 14563-14567

10. Cherfils J., Chabre M., “Activation of G-protein G_α subunits by receptors through G_α - G_β and G_α -G_γ interactions” *TRENDS in Biochemical Sciences* 28 (2003) 13-17

11. Cherfils J., Chardin P., “GEFs: structural basis for their activation of small GTP-binding proteins” *TIBS* 24 (1999) 306-311

12. Danner S., Lohse M., “Phosducin is a ubiquitous G-protein regulator” *PNAS* 93 (1996) 10145-10150

13. De Vries L., Fischer T., TronchereH., Brothers G.M., Strockbine B., Siderovski D. P., Farquhar M.G., “ Activator of G protein signaling 3 is a guanine dissociation inhibitor for $G_{\alpha I}$ subunits” *PNAS* 97 (2000) 14364-14369

13. Hamm H.E., “The many faces of G protein signaling” *J. Biol. Chem.* 273:2 (1998) 669-672

14. Hilgenfeld R., “Regulatory GTPases” *Current Opinion in Structural Biology* 5 (1995) 810-817

15. Houslay M.D.,"G_i-2 at the center of an active phosphorylation/dephosphorylation cycle in hepatocytes: The fine-tuning of stimulatory and inhibitory inputs into adenylate cyclase in normal and diabetic states" *Cell Signalling* 3 (1991) 1-9

16. Hur E.M., Kim K.T., "G protein-coupled receptor signaling and cross-talk Achieving rapidity and specificity" *Cellular Signalling* 14:4 (2002) 397-405

17. Jelsema C.L., Axelrod J. "Stimulation of phospholipase A_2 activity in bovine rod outer segments by the $\beta\gamma$ subunits of transducin and its inhibition by the α subunit" *Proc. Natl. Acad. Sci. USA* 84 (1987) 3623-3627

18. Jones A.M., "G-protein-coupled signaling in *Arabidopsis* " *Current Opinion in Plant Biology* 5 (2002) 402-407

19. Kim M.C., Panstruga R., Elliot C., Müller J., Devoto A., Yoon H.W., Park H.C., Cho M.J., Schulze P., "Calmodulin interacts with MLO protein to regulate defence against mildew in barley" *Nature* 416 (2002) 447-450

20. Legendre L, Heinstein P.F., Low P.S., "Evidence for Participation of GTP-binding Elicitation of the Rapid Oxidative Burst in Cultured Soybean Cells" *J. Biol. Chem*. 267 (1992) 20140-20147

21. Linder M.E., Manahan C.L., Patnana M., Blumer K.J., " Dual lipid modification motifs in G_α and G_γ subunits are required for full activity of the pheromone response pathway in *Sacchoromyces cerevisiae* " *Molecular Biology of the Cell* 11(2000) 957-968

22. Marinissen M.J., Gutkind J.S., "G-protein-coupled receptors and signaling networks: emerging paradigms" *Trends in Pharmocological Sciences* 22:7 (2001) 368-376

23. Merkle T., Neuhaus G., Obrdlik P., " Plant heterotrimeric G protein β subunit is associated with membranes via protein interactions involving coiled-coil formation" *FEBS Letters* 476 (2000) 208-212

24. Peitzsch R.M., McLaughin S., "Binding of acylated peptides and fatty acids to phospholipid vesicles: Pertinence to myristoylated proteins" *Biochemistry* 32 (1993) 10436-10443

25. Rahmatullah M., Ginnan R., Robishaw D., "Specificity of G Protein α-γ Interactions" J. Biol. Chem. 270 (1995) 2946-295

26. Robishaw J.D., Kalman V.K., Moomaw C.R., Slaughter C.A., "Existence of Two γ-Subunits of the G Proteins in Brain" *The Journal of Biological Chemistry* 264:27 (1989) 15758-15761.

27. Schmidt C.J., Thomas T.C., Levine M.A., Neer E.J., "Specificity of G protein β and γ subunit Interactions" *J. Biol. Chem*. 267 (1992) 13807-13810

28. Silvius J.R., l'Heureux F., "Fluorometric Evaluation of the Affinities of Isoprenylated Peptides for Lipid Bilayers" *Biochemistry* 33 (1994) 3014-3022

29. Sprang S.R., "G protein mechanisms: Insights from structural analysis" *Annu. Rev. Biochem.* 66 (1997) 639-678

30. Steitz T.A., Correll C.C., Munishkin A., Chan Y-L., Ren Z., Wool I. G., " Crystal structure of the ribosomal RNA domain essential for binding elongation factors" *PNAS* 95(1998) 13436-13441

31. Tang W.J., Iniguez J.A., Mumby S., Gilman G. " Regulation of mammalian adenylyl cyclases by G-protein α and βγ subunits" *Cold Spring Harbor Symp. Quant. Biol.* 57 (1992) 135-144

32. Wakamatsu K., Tanaka T., Kubota M., Samizo., Nakajima Y., Hoshino M., Kohno T., "One-step affinity purification of the G protein βγ subunits from bovine brain using a histidine-tagged G protein α subunit" *Protein Expression and Purification* 15 (1999) 207-212

33. Walker J.C., Lease K.A., Wen J., Li J., Doke J.T., Liscum E., " A mutant *Arabidopsis* heterotrimeric G-protein β subunit affects leaf, flower, and fruit development" *The Plant Cell* 13 (2001) 2631-2641

34. Wickman K.D., Iniguez-Lluhl J.A., Davenport P.A., Taussig R., Krapivinsky G.B., Linder M.E., Gilman A.G., Clapham D.E. "Recombinant G-protein βγ-subunits activate the muscarinic-gated atrial potassium channel" *Nature* 368 (1994) 255-257

APPENDIX A

A.1 cDNA sequence of AGG1 from TAIR home page

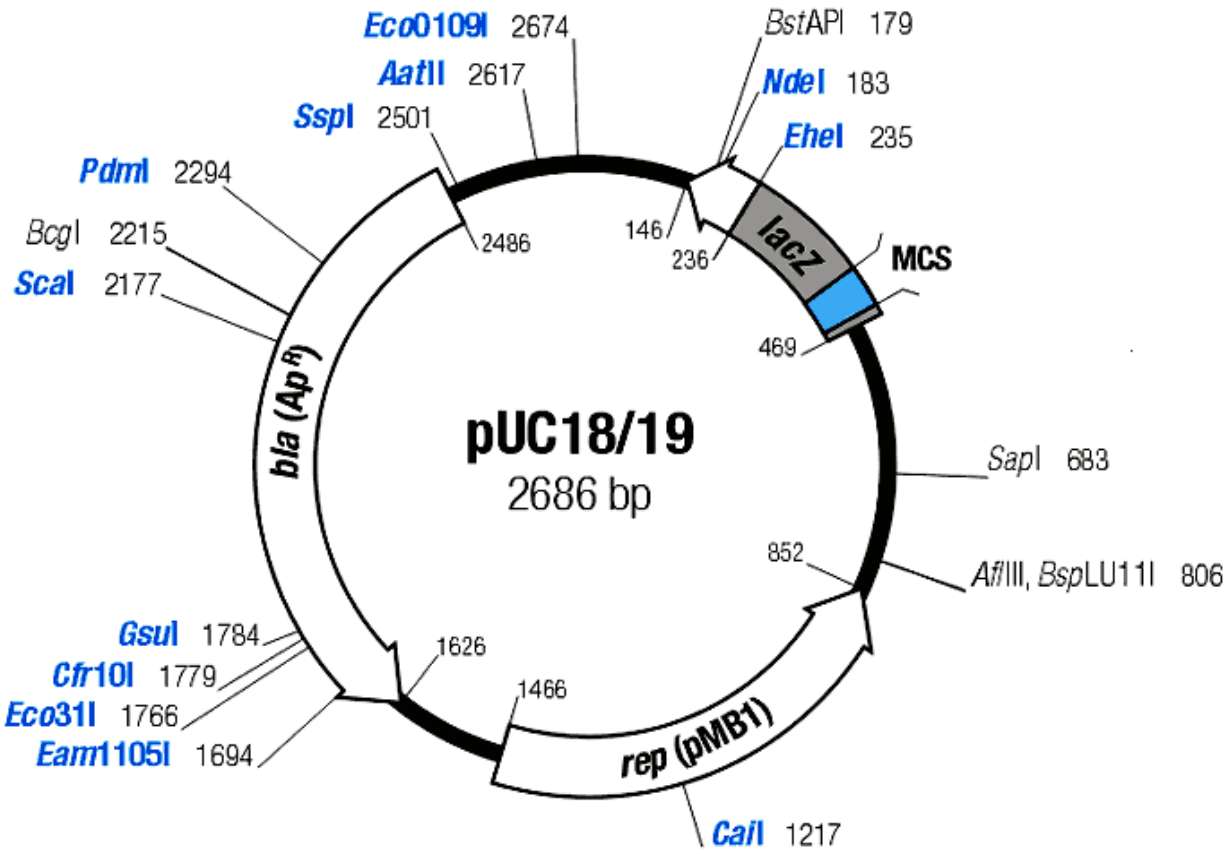
ATGCGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGC
GGGGGCAAGCACAGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTC
GCTTTCTTGGAGAAAGAGTTGAAGGAGGTCGAGAACACAGATATTGTATCA
ACCGTGTGTGAGGAGCTGCTATCTGTCATCGAGAAAGGACCCGATCCTCTGT
TGCCACTAACCAATGGACCTTTGAACTTAGGATGGGACCGGTGGTTTGAAG
GACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTTTGA

A.2 Amino acid sequence of AGG1 from TAIR home page

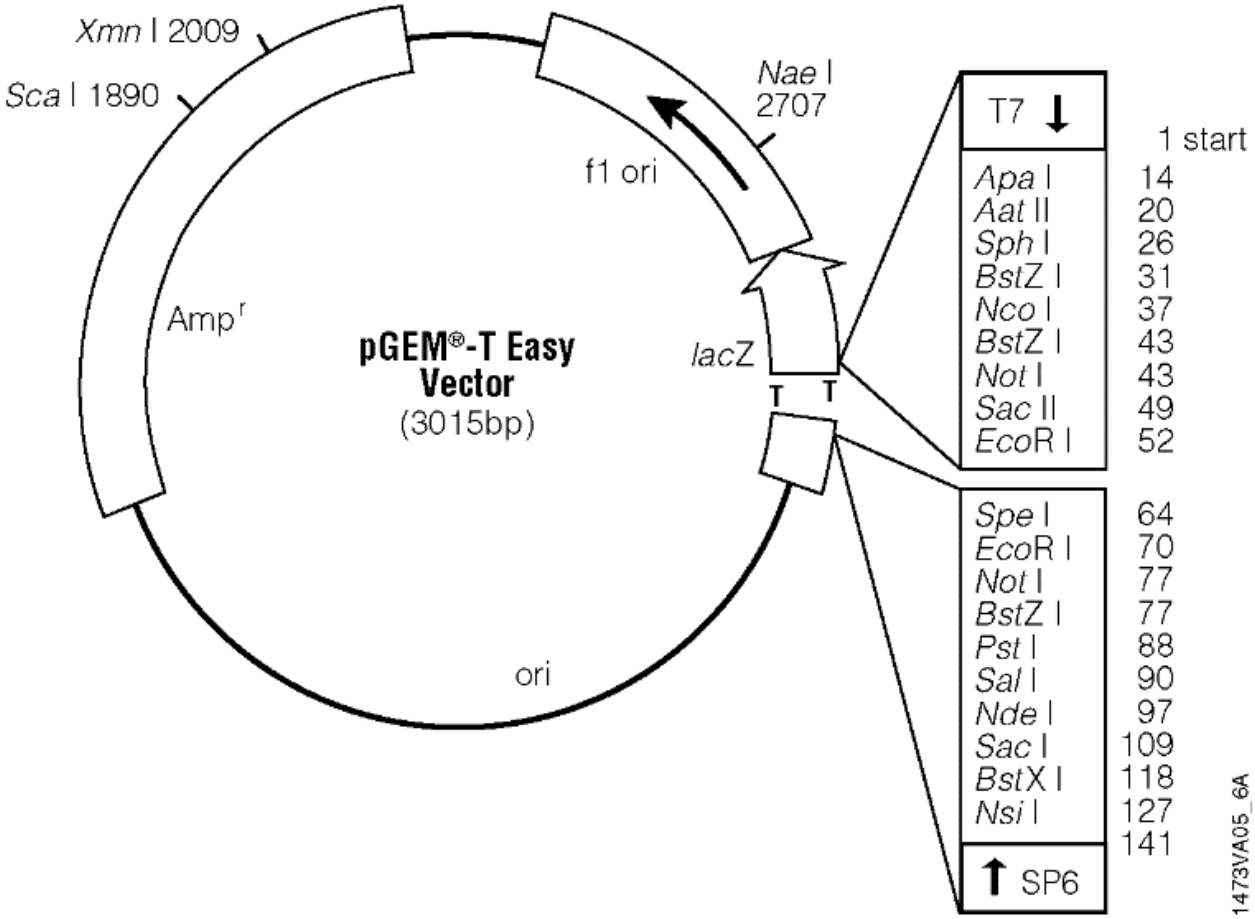
MREETVVYEQEESVSHGGGKHRILAELARVEQEVAFLEKELKEVENTDIVSTVCEELLSVIEKGPDPLLPLTN
GPLNLGWDRWFEGPNGGEGCRCLIL

Met – Arg – Glu – Glu – Thr – Val – Val – Tyr – Glu – Gln – Glu – Glu – Ser – Val – Ser – His – Gly – Gly – Gly – Lys – His – Arg – Ile – Leu – Ala – Glu – Leu – Ala – Arg – Val – Glu – Gln – Glu – Val – Ala – Phe – Leu – Glu – Lys – Glu – Leu – Lys – Glu – Val – Glu – Asn – Thr – Asp – Ile – Val – Ser – Thr – Val – Cys – Glu – Glu – Leu – Leu – Ser – Val – Ile – Glu – Lys – Gly – Pro – Asp – Pro – Leu – Leu – Pro – Leu – Thr – Asn – Gly – Pro – Leu – Asn – Leu – Gly – Trp – Asp – Arg – Trp – Phe – Glu – Gly – Pro – Asn – Gly – Gly – Glu – Gly – Cys – Arg – Cys – Leu – Ile – Leu

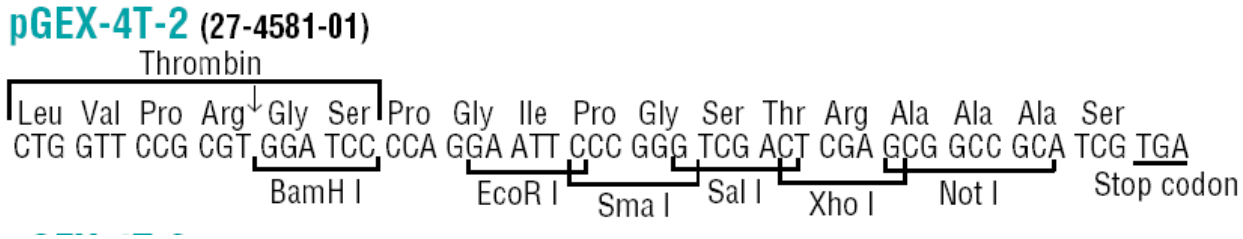
A.3 pUC vector map

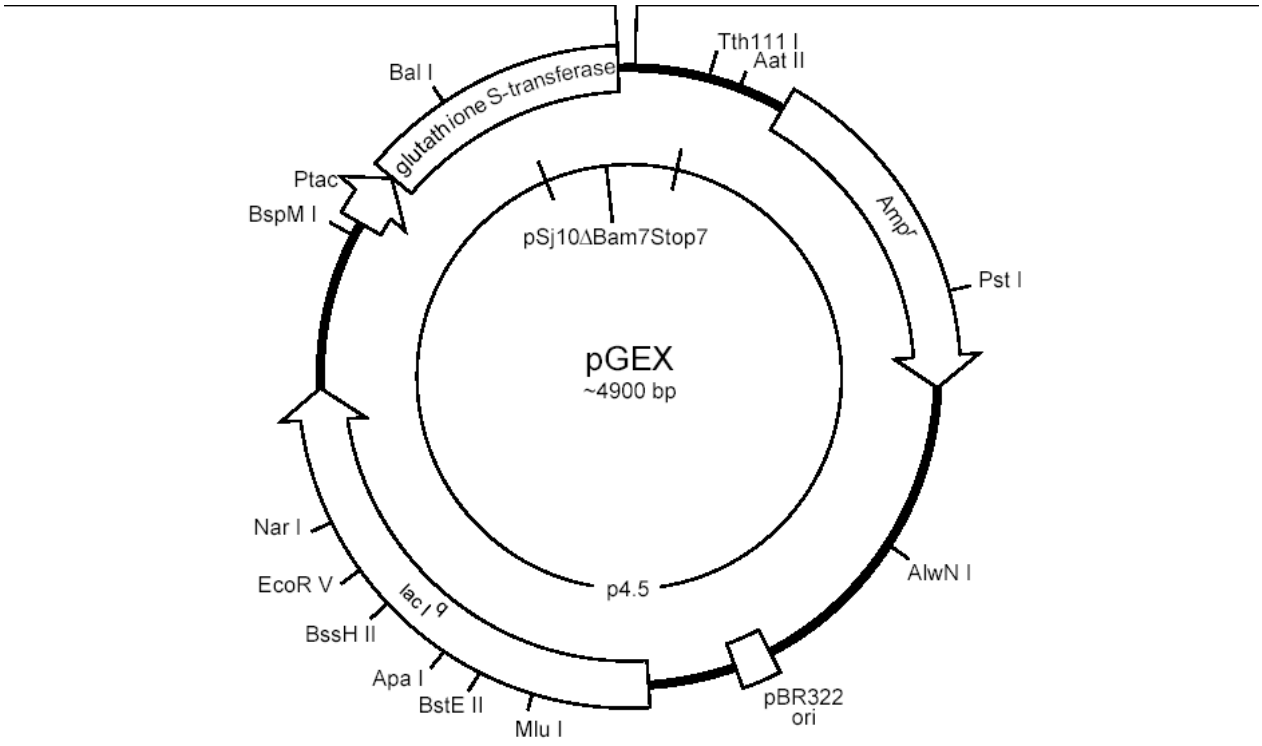

Eco0109I 2674
AatII 2617
SspI 2501
PdmI 2294
BcgI 2215
ScaI 2177
BstAPI 179
NdeI 183
EheI 235
2486
146
236
lacZ
MCS
469
bla (Ap^R)
pUC18/19
2686 bp
SapI 683
852
AflIII, BspLU11I 806
GsuI 1784
Cfr10I 1779
Eco31I 1766
Eam1105I 1694
1626
1466
rep (pMB1)
CaiI 1217

A.4 pGEMTEasy vector circle map and sequence reference points

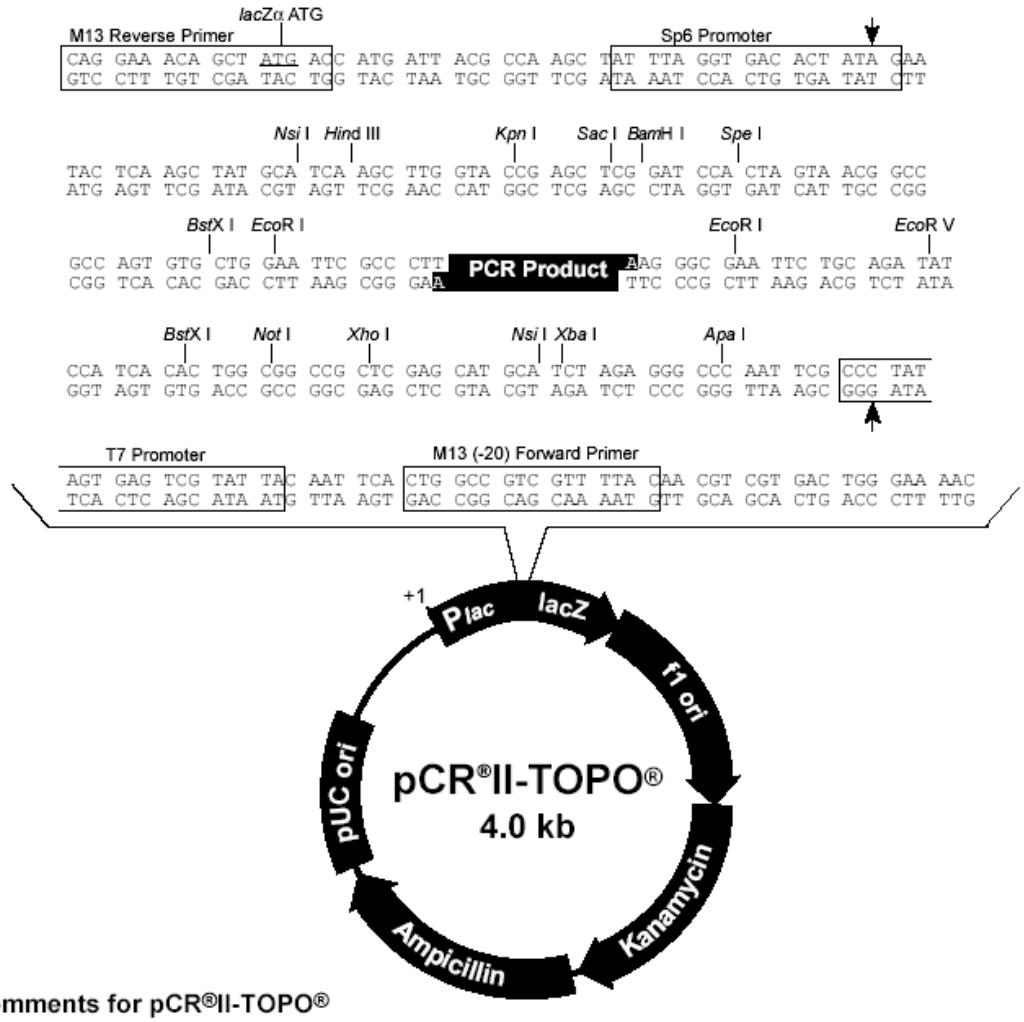


A.5 pGEX-4T2 vector map





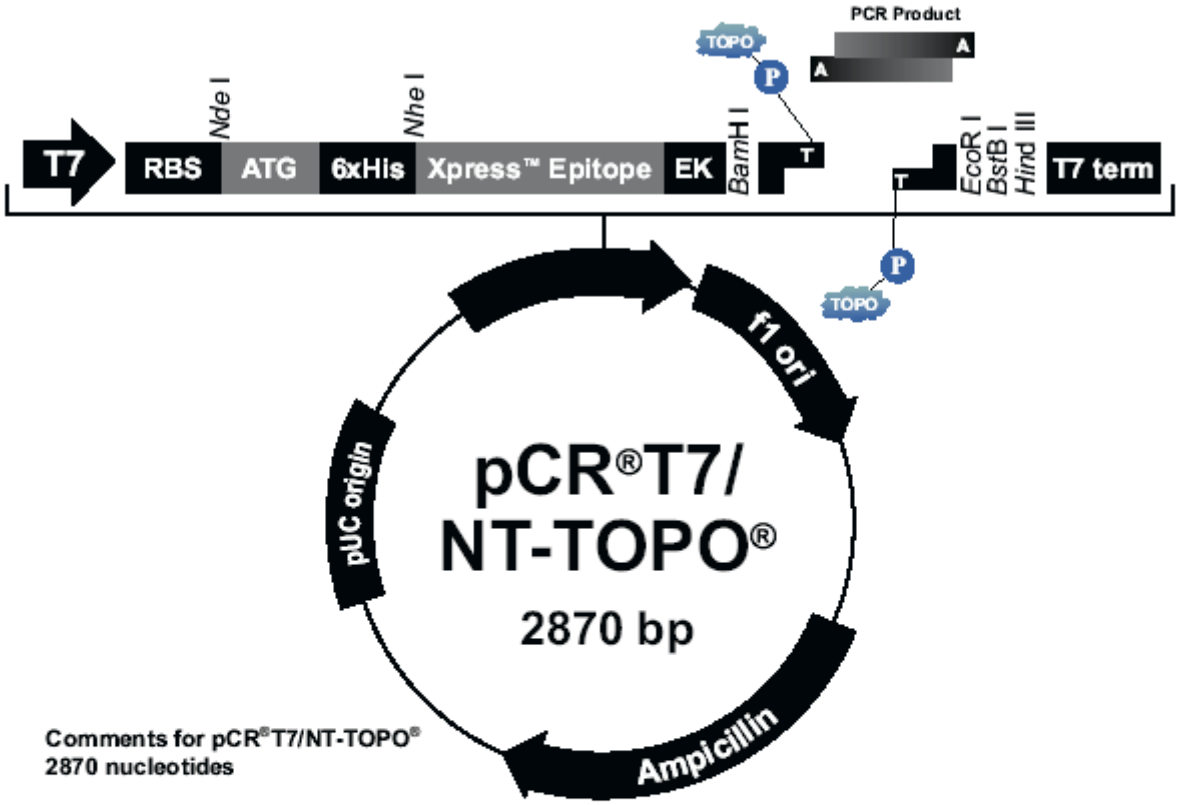
A.6 pCRII-TOPO vector map and sequence reference points



Comments for pCR®II-TOPO®
3973 nucleotides

*Lac*Zα 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

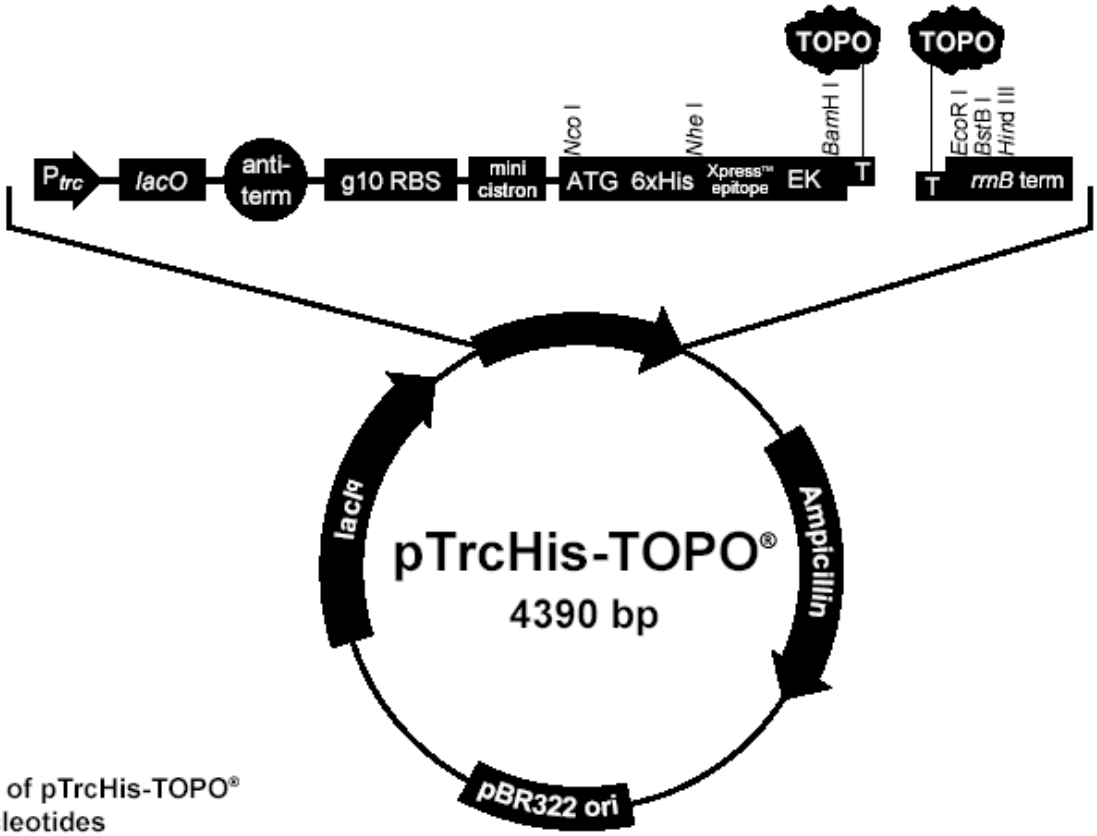
A.7 pCR T7/NT-TOPO vector including multiple cloning site and sequence



Comments for pCR®T7/NT-TOPO®
2870 nucleotides

T7 promoter: bases 20-36
T7 promoter priming site: bases 20-39
Ribosome binding site: bases 87-90
Initiation ATG: bases 100-102
Polyhistidine (6xHis) region: bases 112-129
Xpress™ epitope: bases 169-192
EK recognition site: bases 178-192
TOPO® Cloning site: bases 204-205
T7 reverse priming site: bases 270-289
T7 transcription termination region: bases 231-360
f1 origin: 431-886
Ampicillin resistance gene (ORF): bases 1017-1877
pUC origin: 2022-2695

A.8 pTrcHis-TOPO vector including multiple cloning site and sequence reference points



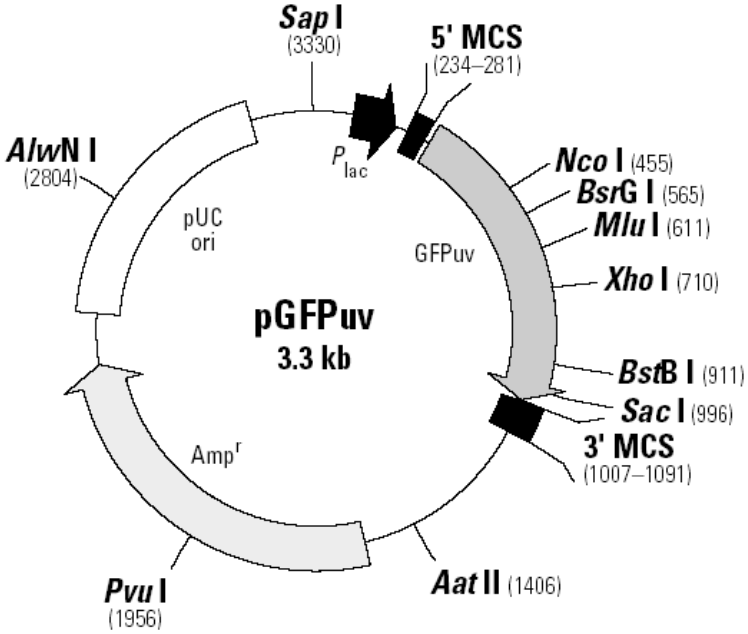
Features of pTrcHis-TOPO®
4390 nucleotides

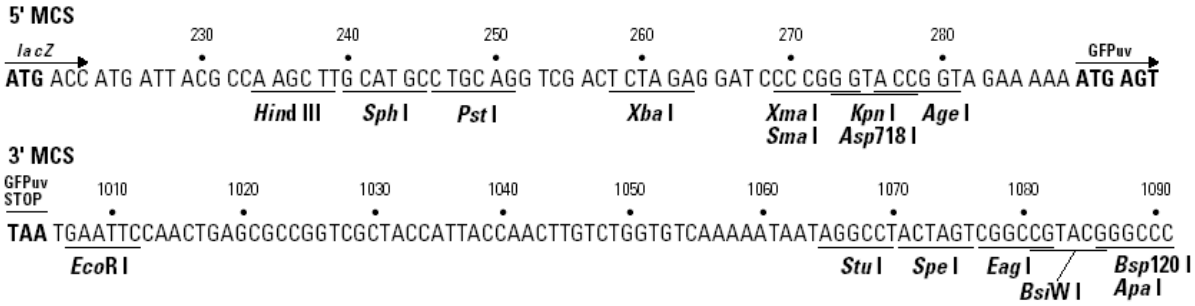
trc promoter and 5' UTR: bases 190-382
-35 region: bases 193-198
-10 region: bases 216-221
lac operator site: bases 228-248
rrnB anti-termination sequence: bases 264-333
T7 gene 10 translational enhancer: bases 346-354
Ribosome binding site: 369-373
pTrcHis forward priming site: bases 370-390
Minicistron: bases 383-409
Reinitiation RBS: bases 398-403
Initiation ATG: bases 413-415
6xHis tag: bases 425-442
Xpress™ epitope: bases 482-505
Xpress™ forward priming site: bases 445-463
Enterokinase cleavage site: bases 491-505
TOPO® Cloning site: bases 517-518
pTrcHis reverse priming site: bases 574-591
rrnB T_1 and T_2 transcription termination sequence: bases 624-781
bla promoter: bases 1002-1059
Ampicillin resistance gene (*bla*): bases 1060-1920
pBR322-derived origin: bases 2065-2738
Lac Repressor ($lacI^q$) : bases 3267-4351

A.9 pGFPuv Vector Map

pGFPuv Vector Information PT3055-5

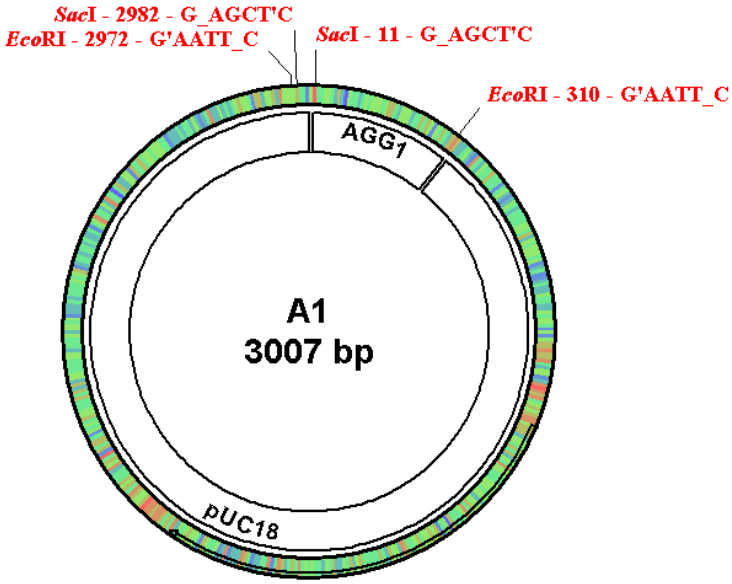
GenBank Accession #U62636 Catalog #6079-1





APPENDIX B

B.1 Map of construct A1 (AGG1 cloned into pUC18 in 5'→ 3' direction)



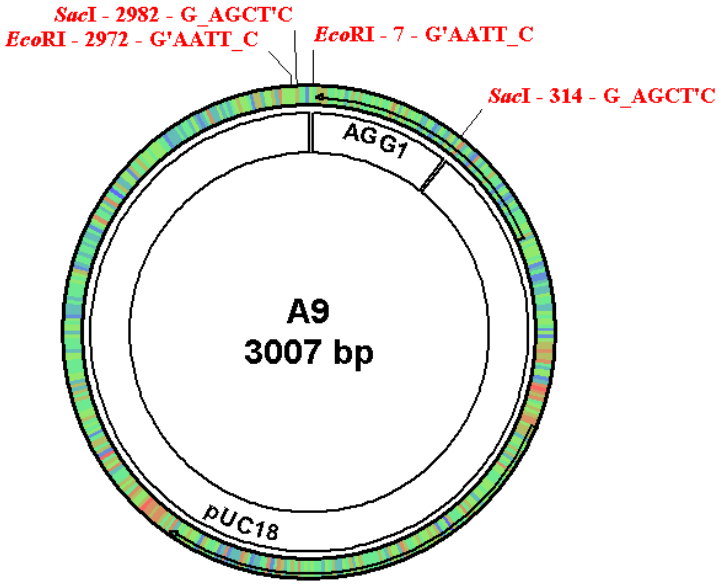
Molecule: AGG1-pUC18, 3007 bps DNA circular

Notes: AGG1 was PCR amplified containing SacI and EcoRI restriction sites and blunt end-ligated into pUC18 plasmid in forward direction.

Enzymes:

SacI	11,	SbfI	329,	NarI	502,	SspI	923
PpuMI	202,	BspMI	332,	SfoI	503,	EcoRI	2972
AgeI	254,	SphI	335,	BbeI	505,	SacI	2982
BsmFI	265,	HindIII	337,	NdeI	554,	KpnI	2988
EcoRI	310,	KasI	501,	AatII	809,	XbaI	2999

B.2 Map of construct A9 (AGG1 cloned into pUC18 in 3'→ 5' direction)



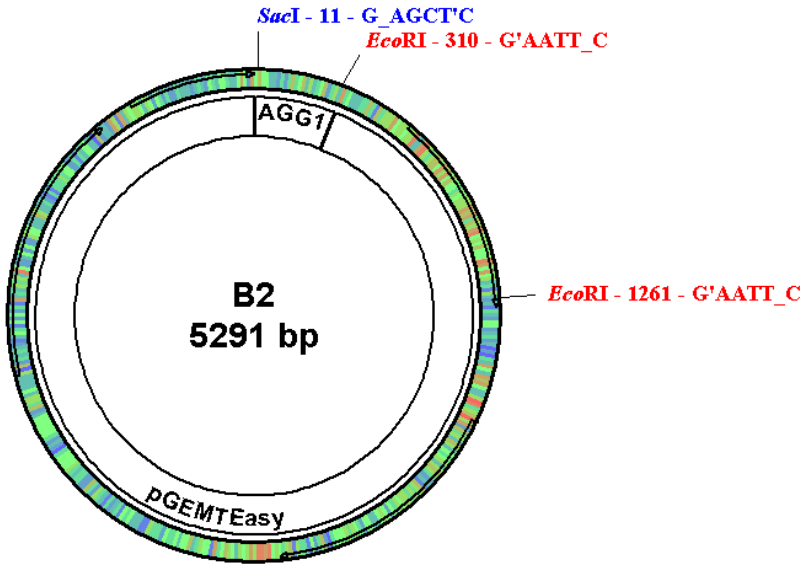
Molecule: AGG1-pUC18, 3007 bps DNA circular

Notes: AGG1 was PCR amplified containing SacI and EcoRI restriction sites and blunt end-ligated into pUC18 plasmid in forward direction.

Enzymes:

EcoRI	7,	SbfI	329,	NarI	502,	SspI	923
BsmFI	52,	BspMI	332,	SfoI	503,	EcoRI	2972
AgeI	63,	SphI	335,	BbeI	505,	SacI	2982
PpuMI	116,	HindIII	337,	NdeI	554,	KpnI	2988
SacI	314,	KasI	501,	AatII	809,	XbaI	2999

B.3 Map of construct B2 (AGG1 + pGEMTEasy)



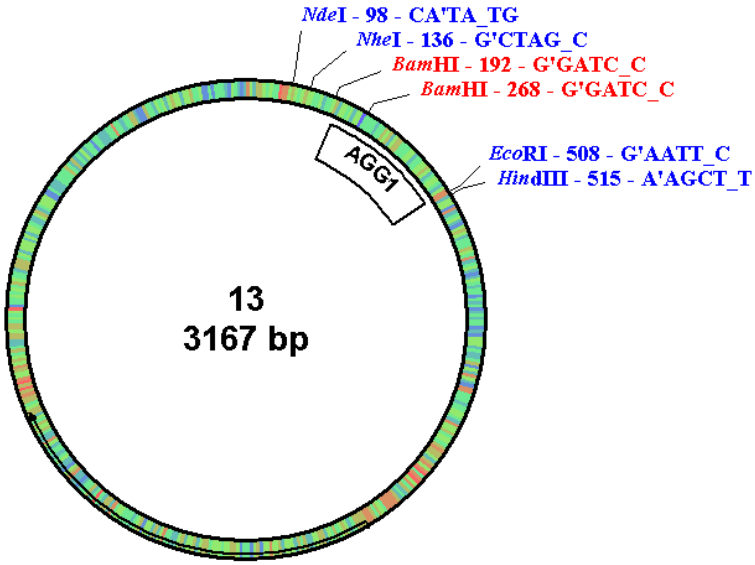
Molecule: AGG1-pGEMTEasy, 5291 bps DNA circular

Notes: AGG1 was PCR amplified containing SacI and EcoRI restriction sites and ligated into pGEMTEasy plasmid.

Enzymes:

SacI	11,	XhoI	1276,	EcoRV	4440
AgeI	254,	NotI	1282,	HpaI	4496
EcoRI	310,	Bsa	2420,	KasI	4629
EcoRI	1261,	AhdI	2486,	NarI	4630
XmaI	1266,	MluI	3990,	SfoI	4631
SmaI	1268,	ApaI	4201,	BbeI	4633

B.4 Map of construct 13 (AGG1 + pT7/NT TOPO)



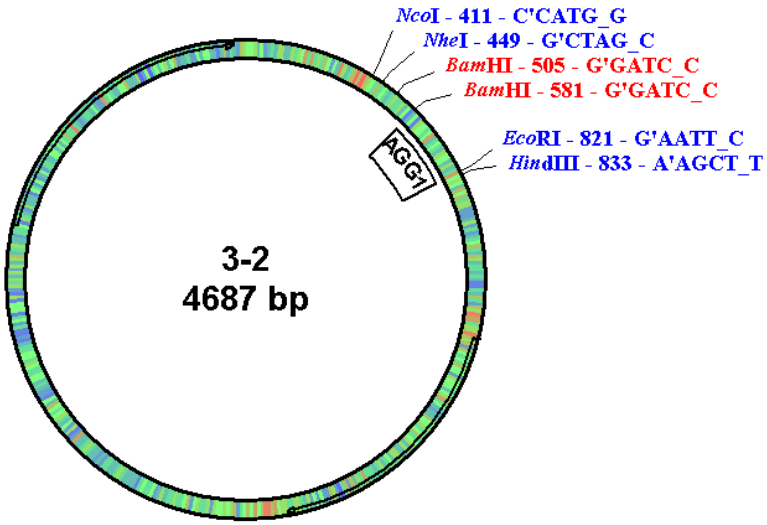
Molecule: AGG1-pCRT7, 3167 bps DNA circular

Notes: AGG1 was PCR amplified containing no restriction sites and ligated into pCRT7
plasmid.

Enzymes:

NsiI	1,	NdeI	395,	XmnI	1501,	SapI	3106
SphI	3,	BamHI	489,	ScaI	1620,		
BamHI	65,	EcoRI	508,	BpmI	2032,		
PstI	280,	HinIII	515,	BmrI	2061,		
XbaI	355,	PsiI	1086,	PciI	2989,		

B.5 Map of construct 3-2 (AGG1 + pTrcHisTOPO)



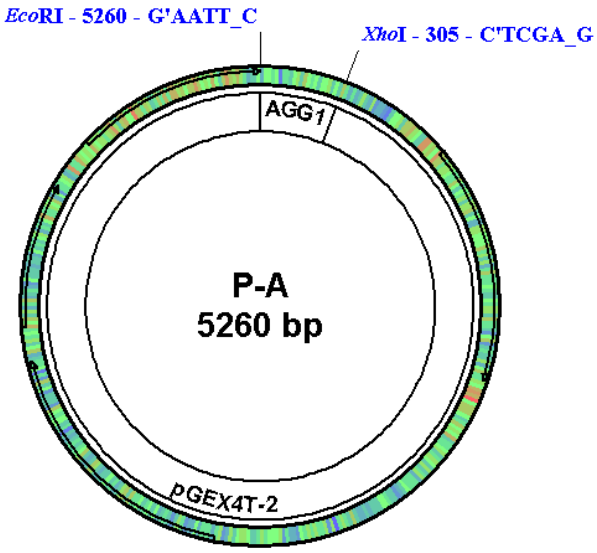
Molecule: AGG1-pTrcHisTOPO, 4687 bps DNA circular

Notes: AGG1 was PCR amplified containing no restriction sites and ligated into pTrcHisTOPO plasmid.

Enzymes:

BamHI 65,	BamHI 802,	SapI 3149,	EcoRV 4366
AgeI 242,	EcoRI 821,	NdeI 3211,	KasI 4555
PstI 280,	HindIII 833,	SphI 3477,	SfoI 4557
NcoI 708,	PvuI 1775,	MluI 3916,	
NheI 746,	PciI 3032,	ApaI 4127,	

B.6 Map of construct P-A (AGG1 + pGEX4T-2)



Molecule: AGG1-pGEX4T2, 5260 bps DNA circular

Notes: AGG1 was excised from PCRII-TOPO using EcoRI and XhoI and ligated into
pGEX4T-2 plasmid which was digested with the same enzymes.

Enzymes:

BamHI	72,	SapI	2520,	SapI	4657,	EcoRI	5260
PstI	287,	BclI	3033,	PciI	4816,	BamHI	5250,
XhoI	305,	EcoRV	3469,	XmnI	4971,	SspI	710,
NotI	311,	HpaI	3525,	ScaI	5151,	SspI	4486,

APPENDIX C

All the sequence alignments in the Appendix C were done using SDSC Biology Workbench.

8. C.1 Sequence alignment of AGG1-pBS with AGG1 to verify the sequence of AGG1

```
CLUSTAL W (1.81) multiple sequence alignment


AGG1*  ------------------------------------------------------------  60
AGG1   CGGGCCCCCCCCTCGAGATTTACCCCAGACTCTCTCTGACGTTGTCAGATCTGCGACCTGC


AGG1*  ----------ATGCGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGG 120
AGG1   TAGCGAGAGGATGCGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGG
                 ************************************************


AGG1*  CGGGGGCAAGCACAGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTT 180
AGG1   CGGGGGCAAGCACAGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTT
       ************************************************************


AGG1*  GGAGAAAGAGTTGAAGGAGGTCGAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCT 240
AGG1   GGAGAAAGAGTTGAAGGAGGTCGAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCT
       ************************************************************
```

```
AGG1* GCTATCTGTCATCGAGAAAGGACCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAA 300
AGG1  GCTATCTGTCATCGAGAAAGGACCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAA
      ************************************************************

AGG1* CTTAGGATGGGACCGGTGGTTTGAAGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAAT 360
AGG1  CTTAGGATGGGACCGGTGGTTTGAAGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAAT
      ************************************************************

AGG1* ACTTTGA---------------------------------------------------- 420
AGG1  ACTTTGATTCGCTAGACAGAGTAATGTGTTACATATACTAGTTTCTTGCTAGGATATCAT
      *******

AGG1* ------------------------------------------------------------ 480
AGG1  TTTACATGGGATCCAAAATGGGGCTGTAGCAGAGAAAAAGCGTACAGATTAATGAGCATT


AGG1* ------------------------------------------------------------ 540
AGG1  GGAATTGCATAGAACGGCTTAGTTGCACCTACGTAAACAGTATTATATGTATATGTAATG


AGG1* ------------------------------------------------------------ 600
AGG1  TATGGGATCCGGATCTGACATACGAGTAATGGAGTTTCAGGCTTACAGCTAAAAAAAAAG


AGG1* ------------------------------------------------------------ 660
AGG1  GTAAATCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGA


AGG1* ----------------------------------------
AGG1  TCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCC
```

C.2 Sequencing result of A1up (AGG1 cloned into pUC18 in 5'→ 3' direction)

```
CLUSTAL W (1.81) multiple sequence alignment


A1up  GTAACACGGCCGTGCCAGCTTGCATGCCTGCAGGTCTATATAGAGCTCATGCGAGAGGAA 60
AGG1* ------------------------------------------------ATGCGAGAGGAA
                                                         ************


A1up  ACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGGGGCAAGCACAGGATCCTT 120
AGG1* ACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGGGGCAAGCACAGGATCCTT
      ************************************************************

A1up  GCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAGAAAGAGTTGAAGGAGGTC 180
AGG1* GCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAGAAAGAGTTGAAGGAGGTC
      ************************************************************

A1up  GAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTATCTGTCATCGAGAAAGGA 240
AGG1* GAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTATCTGTCATCGAGAAAGGA
      ************************************************************

A1up  CCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTAGGATGGGACCGGTGGTTT 300
AGG1* CCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTAGGATGGGACCGGTGGTTT
```

```
      ************************************************************

A1up  GAAGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTTTGAGAATTCCTGTGCGAC 360
AGG1* GAAGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTTTGA--------------
      *******************************************

A1up  TCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATCATGGTCATAGCTGTTTCCTGT 420
AGG1* ------------------------------------------------------------


A1up  GTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAA 480
AGG1* ------------------------------------------------------------


A1up  AGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGC 540
AGG1* ------------------------------------------------------------


A1up  TTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAG 600
AGG1* ------------------------------------------------------------


A1up  AGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGT 660
AGG1* ------------------------------------------------------------


A1up  CGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGA 720
AGG1* ------------------------------------------------------------


A1up  ATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGNCAAAGGCCAGGAACCG 780
AGG1* ------------------------------------------------------------


A1up  TNAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAA 840
AGG1* ------------------------------------------------------------


A1up  AAATCGACGCTCNAGTCAGAAGTGGCGAAACCCGACAGGACTATAANGATACCAGGCGTT 900
AGG1* ------------------------------------------------------------


A1up  TCCC
AGG1* ----
```

C.3 Sequencing result of A9down (AGG1 cloned into pUC18 in 3'→ 5' direction)

```
CLUSTAL W (1.81) multiple sequence alignment


A9downACGCTATGACCTTGATTACGAATTCGAGCTCCGTCCCGGGGATCCTCTAGAGTCTATATA  60
AGG1* ------------------------------------------------------------
```

```
A9downGAGCTCATGCGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGG 120
AGG1* ------ATGCGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGG
            ************************************************************

A9downGGCAAGCACAGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAG 180
AGG1* GGCAAGCACAGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAG
      ************************************************************

A9downAAAGAGTTGAAGGAGGTCGAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTA 240
AGG1* AAAGAGTTGAAGGAGGTCGAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTA
      ************************************************************

A9downTCTGTCATCGAGAAAGGACCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTA 300
AGG1* TCTGTCATCGAGAAAGGACCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTA
      ************************************************************

A9downGGATGGGACCGGTGGTTTGAAGGACCAAATGGAGGAGAAGGCCGCAGATGCTTAATACTT 360
AGG1*GGATGGGACCGGTGGTTTGAAGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTT
     ***************************************** ****************

A9downTGAGAATTCCTGTGCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACG 420
AGG1* TGA-----------------------------------------------------------
      ***

A9downTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTT 480
AGG1* ------------------------------------------------------------


A9downCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAG 540
AGG1* ------------------------------------------------------------


A9downCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTC 600
AGG1* ------------------------------------------------------------


A9downACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCC 660
AGG1* ------------------------------------------------------------


A9downCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGC 720
AGG1* ------------------------------------------------------------


A9downTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATC 780
AGG1* ------------------------------------------------------------


A9downACCGAAACGCGCGAGACGANAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCAT 840
AGG1* ------------------------------------------------------------


A9downGATAATAATGGTTTCTTAGACGTCNAGTGGCACTTTTTCGGGGAATGTGCGCGGAAC
AGG1* ------------------------------------------------
```

C.4 Sequencing result of B2 (pGEMTEasy+AGG1)

```
CLUSTAL W (1.81) multiple sequence alignment

B2     AATGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTTATATA  60
AGG1* ------------------------------------------------------------

B2     GAGCTCATGCGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGG 120
AGG1* ------ATGCGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGG
             ************************************************************

B2     GGCAAGCACAGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAG 180
AGG1* GGCAAGCACAGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAG
      ************************************************************

B2     AAAGAGTTGAAGGAGGTCGAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTA 240
AGG1* AAAGAGTTGAAGGAGGTCGAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTA
      ************************************************************

B2     TCTGTCATCGAGAAAGGACCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTA 300
AGG1* TCTGTCATCGAGAAAGGACCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTA
      ************************************************************

B2     GGATGGGACCGGTGGTTTGACGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTT 360
AGG1* GGATGGGACCGGTGGTTTGAAGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTT
      ******************* ****************************************

B2     TGAGAATTCCTGTGCAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGA 420
AGG1* TGA---------------------------------------------------------
      ***

B2     GAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTG 480
AGG1* ------------------------------------------------------------

B2     GCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACAC 540
AGG1* ------------------------------------------------------------

B2     AACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTC 600
AGG1* ------------------------------------------------------------

B2     ACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTG 660
AGG1* ------------------------------------------------------------

B2     CATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCT 720
AGG1* ------------------------------------------------------------

B2     TCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCNA 780
AGG1* ------------------------------------------------------------

B2     CTCAAGGCNGGTATACGGTTATCCACAGAATCAGGNNGATACGCANGAAAAGACATGTGA 840
```

```
AGG1* ------------------------------------------------------------

B2    GCANAGGCCAGCANNAGGGCAGAACCCGTAAAGGGCGCGTGCTGGCGTTTTCCCATAGCT 900
AGG1* ------------------------------------------------------------

B2    CCGCCCCCTG
AGG1* ----------
```

C.5 Sequencing result of 13 (pT7/NT TOPO expression+AGG1)

```
CLUSTAL W (1.81) multiple sequence alignment

13    ANCCAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG  60
AGG1* ------------------------------------------------------------

13    CGGGGTTCTCATCATCATCATCATCATGGTATGGCTAGCATGACTGGTGGACAGCAAATG 120
AGG1* ------------------------------------------------------------

13    GGTCGGGATCTGTACGACGATGACGATAAGGATCCAACCCTTCAAAGTATTAAGCATCTG 180
AGG1* ------------------------------------------------------------

13    CAGCCTTCTCCTCCATTTGGTCCTTCAAACCACCGGTCCCATCCTAAGTTCAAAGGTCCA 240
AGG1* ---------------------------------------------ATGCGAGAGGAAACT
                                                     * *   * *   *

13    TTGGTTAGTGGCAACAGAGGATCGGGTCCTTTCT-CGATGACAGATAGCAGCTCCTCACA 300
AGG1* GTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGGGGCAAGCACAGGATCCTTGCA
       *****   *   *    *  ** * * **  *  **  * **   *   * ****  **

13    CACGGTTGATACAATATCTGTGTTCTCGACCTCCTTCAACTCTTTCTCCAAGAAAGCGAC 360
AGG1* GAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAGAAAGAGTTGAAGGAGGTCGAG
       *   *     *  *      *   ***   ** *  *        *  * **   ***

13    TTCCTGTTCAACGCGGGCAAGCTCTGCAAGGATCCTGTGCTTGCCCCCGCCGTGAGAAAC 420
AGG1* AACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTATCTGTCATCGA-GAAAGGACC
      *      *  *     *   * **  **** *   *   ** *  **  *  ** * *

13    AGACTCCTCCTGCTCGTAAACCACAGTTTCCTCTCGCATAAGGGCGAATTCGAAGCTTGA 480
AGG1* CGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTAGGATGGGACCGGTGGTTTGA
      **  *    *   *   *****  *   * *    * ** *   * *   *  * ****

13    TCCGGCTGCTAACAAAGCCCGAAAGGAAGCTG-AGTTGGCTGCTGCCACCGCTGAGCAAT 540
AGG1* AG--------GACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTTTGA---------
```

```
      ** **       ** * **** ** **  *  * *

13    AACTAGCATAACCCCTTGGGGCCTCTAAACGGG
AGG1* --------------------------------
```

C.6 Sequencing result of 3-2 (pTrcHisTOPO+AGG1)

```
CLUSTAL W (1.81) multiple sequence alignment


3-2   TNATANGGAGGAATNAATTNTGGGGGGTTNTCTCATCTCTCATCATGGTATGGCTAGCAT  60
AGG1* ------------------------------------------------------------


3-2   GACTGGTGGACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGGATCCAACCCT 120
AGG1* ------------------------------------------------------------


3-2   TATGCGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGGGGCAA 180
AGG1* -ATGCGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGGGGCAA
       ************************************************************


3-2   GCACAGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAGAAAGA 240
AGG1* GCACAGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAGAAAGA
      ************************************************************


3-2   GTTGAAGGAGGTCGAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTATCTGT 300
AGG1* GTTGAAGGAGGTCGAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTATCTGT
      ************************************************************


3-2   CATCGAGAAAGGACCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTAGGATG 360
AGG1* CATCGAGAAAGGACCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTAGGATG
      ************************************************************


3-2   GGACCGGTGGTTTGAAGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTTTGAAA 420
AGG1* GGACCGGTGGTTTGAAGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTTTGA--
      ************************************************************


3-2   GGGCGAATTCGAAGCTTGGCTGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGATACAGA 480
AGG1* ------------------------------------------------------------


3-2   TTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGG 540
AGG1* ------------------------------------------------------------


3-2   TGGTCCCACCTGACCCCATGCCGAACTCAGAAGTG
AGG1* ----------------------------------
```

C.7 Sequencing result of P-A (pGEX4T-2+AGG1)

```
CLUSTAL W (1.81) multiple sequence alignment

P-A    TGGGCGGACCATCCCTCCAAAATCGGATCTGGTTCCGCGTGGATCCCCAGGAATTCCCTG  60
AGG1* ------------------------------------------------------------ATG
                                                                   **

P-A    CGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGGGGCAAGCAC 120
AGG1* CGAGAGGAAACTGTGGTTTACGAGCAGGAGGAGTCTGTTTCTCACGGCGGGGGCAAGCAC
      ************************************************************

P-A    AGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAGAAAGAGTTG 180
AGG1* AGGATCCTTGCAGAGCTTGCCCGCGTTGAACAGGAAGTCGCTTTCTTGGAGAAAGAGTTG
      ************************************************************

P-A    AAGGAGGTCGAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTATCTGTCATC 240
AGG1* AAGGAGGTCGAGAACACAGATATTGTATCAACCGTGTGTGAGGAGCTGCTATCTGTCATC
      ************************************************************

P-A    GAGAAAGGACCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTAGGATGGGAC 300
AGG1* GAGAAAGGACCCGATCCTCTGTTGCCACTAACCAATGGACCTTTGAACTTAGGATGGGAC
      ************************************************************

P-A    CGGTGGTTTGAAGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTTTGACTCGAG 360
AGG1* CGGTGGTTTGAAGGACCAAATGGAGGAGAAGGCTGCAGATGCTTAATACTTTGA------
      **************************************************

P-A    CGGCCGCATCGTGACTGACTGACGATCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAAC 420
AGG1* ------------------------------------------------------------


P-A    CTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGC 480
AGG1* ------------------------------------------------------------


P-A    AGACAAGCCCGTCAGGGCGCGTCANCGGGTGTTGGCGGGNTGCGGGGCGCAGCCATGACC 540
AGG1* ------------------------------------------------------------


P-A    CAGTCACGTAGCGATAGCGGAGTGTATAATTC
AGG1* --------------------------------
```

APPENDIX D

Aotuclave:	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
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 °C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY
	-20 °C, Bosch, TÜRKİYE
Distilled water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
Gel Documentation:	UVITEC, UVIdoc Gel Documentation System, UK
	Biorad, UV-Transilluminator 2000, USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Model 300, GERMANY

	Memmert, Model 600, GERMANY
Laminar Flow:	Kendro Lab. Prod., Heraus, HeraSafe HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Hetaing Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Metler Toledo, Volumate, USA
Microwave oven:	Bosch, TÜRK□YE
pH meter:	WTW, pH540 GLP Multical®, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
	Wealtec, Elite 3000, USA
Refrigerator:	+4ºC, Bosch, TÜRK□YE
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA

	New Brunswick Sci., Innova™ 4330, USA
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
Waterbath:	Huber, Polystat cc1, GERMANY