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AKDENIZ UNIVERSITY

**EXPRESSION, PRODUCTION AND CHARACHTERISATION OF PLANT
PRODUCED DEGLYCOSYLATED Pfs48/45 VARIENTS**

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INSTITUTE OF NATURAL AND APPLIED SCIENCES

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MASTER'S DEGREE THESIS

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Bu tez 02/07/18 tarihinde jüri tarafından Oybirliği ile kabul edilmiştir.

Prof. Dr. Tarlan MAMMEDOV (Danışman)
Prof. Dr. Yaşar KARAKURT
Dr. Öğretim Görevlisi Münevver AKSOY

ABSTRACT

Expression, production and characterization of plant produced deglycosylated Pfs48/45 variants

Burcu GÜLEÇ

Master's Degree Thesis, Agricultural Biotechnology Department

Supervisor: Prof. Dr. Tarlan MAMMEDOV

May 2018; 35 pages

Plasmodium falciparum is one of the deadliest parasite in human history and therefore, developing safe, low-cost and highly efficient malaria vaccine with long-term stability is urgently needed. Many recent studies have shown that the plant-based transient expression system is most promising technology that enables safe, rapid and inexpensive production of valuable, recombinant proteins with high expression capacity. Pfs48/45 is one of the leading candidates for transmission blocking (TB) vaccine development and plays a key role in parasite fertilization. Plant produced Pfs48/45 of *P. falciparum* could be a great potential as a TB vaccine candidate for a safe, effective, and low-cost vaccine against malaria. Pfs48/45 protein has seven potential glycosylation sites and is aberrantly glycosylated when expressed in *Nicotiana benthamiana* plants. Recently, to avoid the aberrant glycosylation, a strategy was developed to produce non-glycosylated proteins in plants by co-expression of target proteins of interest with bacterial enzymes PNGase F or Endo H (Mamedov et al. 2013, 2016, 2017). In this study, non-glycosylated variants of Pfs48/45 protein, was engineered, expressed and produced in *N. benthamiana* plant using PNGase F or Endo H in vivo deglycosylation strategy. Glycosylated and deglycosylated variants of Pfs48/45 were purified and purified protein were characterized using a conformational specific Pfs48/4 monoclonal antibody (MRA-26, IIC5-B10), a known TB antibody. We demonstrate that, in contrast to the glycosylated form, plant produced *in vivo* Endo H deglycosylated Pfs48/45 was recognized by conformational specific Pfs48/45 monoclonal antibody, in a manner like its PNGase F deglycosylated counterpart. This suggest that plant produced Pfs48/45 protein contain epitopes present on native Pfs48/45 and therefore, have a great potential for the development of a Pfs48/45-based TB malaria vaccine.

KEYWORDS: *Plasmodium falciparum*, Pfs48/45**,** deglycosylation, Endo H*,* PNGase F, transient expression, agroinfiltration, recombinant protein, protein production, disulfide bridges, *Nicotiana benthamiana.*

COMMITTEE: Prof. Dr. Tarlan MAMMEDOV
                  Prof. Dr. Yaşar KARAKURT
                  Dr. Öğr. Üyesi Münevver AKSOY

ÖZET

Bitkiden üretimli deglikozile Pfs48 / 45 varyantlarının ifadesi, üretimi ve karakterizasyonu

Burcu GÜLEÇ

Yüksek Lisans Tezi, Tarımsal Biyoteknoloji Bölümü

Danışman: Prof. Dr. Tarlan MAMMEDOV

Mayıs 2018; 35 sayfa

Plasmodium falciparum, insanlık tarihindeki en ölümcül parazitlerden biridir ve bu nedenle, uzun vadeli stabiliteye sahip güvenli, düşük maliyetli ve yüksek verimli sıtma aşısı geliştirmeye acilen ihtiyaç vardır. Son zamanlardaki birçok çalışma, bitki bazlı geçici ekspresyon sisteminin, yüksek ekspresyon kapasitesine sahip değerli, rekombinant proteinlerin güvenli, hızlı ve ucuz üretimini sağlayan en ümit verici teknoloji olduğunu göstermiştir. Pfs48 / 45, bulaşma engelleme (TB) aşısının geliştirilmesinde önde gelen adaylardan biridir ve parazit fertilizasyonunda önemli bir rol oynar. Bitkide üretilmiş *P. falciparum*'un Pfs48 / 45 proteini, sıtmaya karşı güvenli, etkili ve düşük maliyetli bir aşı için TB aşısı adayı olarak büyük bir potansiyel olabilir. Pfs48 / 45 proteininin yedi potansiyel glikosilasyon bölgesi vardır ve *Nicotiana benthamiana* bitkilerinde eksprese edildiğinde anormal şekilde glikosile edilir. Son zamanlarda, anormal glikosilasyonu önlemek için, bakteriyel enzimler PNGase F veya Endo H ile ilgili hedef proteinlerin birlikte sentezlenmesiyle bitkilerde glikosile edilmemiş proteinlerin üretilmesi için bir strateji geliştirilmiştir (Mamedov vd. 2013, 2016, 2017). Bu çalışmada, Pfs48 / 45 proteininin glikosile edilmemiş varyantları, *in vivo* deglikosilasyon stratejisinde PNGase F veya Endo H kullanılarak *N. benthamiana* bitkisinde tasarlanmış, ifade edilmiş ve üretilmiştir. Pfs48 / 45'in glikosile edilmiş ve deglikosile edilmiş varyantları saflaştırılmış ve saflaştırılmış protein, bilinen bir TB antikoru olan bir konformasyonel spesifik Pfs48 / 45 monoklonal antikoru (MRA-26, IIC5-B10) kullanılarak karakterize edilmiştir. Glikosile edilmiş formun tersine, *in vivo* Endo H deglikosile edilmiş Pfs48 / 45'te üretilen bitkinin, PNGase F deglikosile edilmiş muadiline benzer bir şekilde, konformasyonel spesifik Pfs48 / 45 monoklonal antikoru tarafından tanındığını göstermekteyiz. Bu, bitki tarafından üretilen Pfs48 / 45 proteininin doğal Pfs48 / 45 üzerinde bulunan epitopları içerdiğini ve bu nedenle bir Pfs48 / 45 bazlı TB sıtma aşısının geliştirilmesi için büyük bir potansiyele sahip olduğunu göstermektedir.

KEYWORDS: *Plasmodium falciparum*, Pfs48/45**,** deglycosylation, Endo H, PNGase F, transient expression, agroinfiltration, recombinant protein, protein production, disulfide bridges, *Nicotiana benthamiana*.

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PREFACE

CONTENTS

AKADEMİK BEYAN

Yüksek Lisans Tezi olarak sunduğum "EXPRESSION, PRODUCTION AND CHARACHTERISATION OF PLANT PRODUCED DEGLYCOSYLATED Pfs48/45 VARIANTS" adlı bu çalışmanın, akademik kurallar ve etik değerlere uygun olarak bulunduğunu belirtir, bu tez çalışmasında bana ait olmayan tüm bilgilerin kaynağını gösterdiğimi beyan ederim.

31/05/2018

Burcu Güleç

SYMBOLS and ABBREVIATIONS

<u>Symbols</u>

µL	Microliter
µg	microgram
%	Percent
min.	Minutes
G	Gram
Kb	Kilobase
mL	Milliliter
s	Second
V	Volt
°C	Celsius degrees
x g	Earth's gravitational force
mM	MiliMolar
mG	MiliGram
M	Molar

<u>Abbreviations</u>

DNA	Deoksiribonucleic acid
PCR	Polimerase Chain Reaction
RNA	Ribonucleic acid
pH	Power of Hydrogen
TB	Transmission Blocking
SDS-PAGE	Sodium dodecyl sulfate- Polyacrylamide Gel Electrophoresis
mAbs	Monoclonal Antibodies

LIST OF FIGURES

1. INTRODUCTION

Malaria is a disease of worldwide distribution and is probably the most widespread of all disease. It is caused by a protozoan parasite belonging to the genus *Plasmodium* that are transmitted to people through the bites of infected female Anopheles mosquitoes called malaria vectors. Humans are infected by five common species, the distribution of which is not uniform. *Plasmodium falciparum* cause the most severe form of the disease, known as pernicious, subtertian, malignant or estivoautumnal malaria.

The natural reservoir of the disease is humans. The female Anopheles mosquito transmit the disease by biting an infected person when taking the blood meal and about one week later, when the mosquito takes another blood meal from a healthy person, the parasites injected into the person being bitten through mosquito's saliva.

According to World Health Organization (WHO), In 2016, there were an approximately 216 million cases of malaria in 91 countries and malaria deaths reached 445 000 (Anonymous 1). *Plasmodium falciparum* is responsible for most of deaths and fighting with it is very difficult. For preventing the transmission, anti-malarial drugs and vector control is the main way but a successful vaccine has not developed yet.

Antimalarial drugs can prevent the disease through chemoprophylaxis, which suppresses the blood stage of malaria infections, but drug resistance is a recurring problem. And vector control for preventing and reducing the cases is not effective. A vaccine against malaria needed for eradication of malaria.

Since the *Plasmodium falciparum* is the main responsible malaria parasite of the most of deaths, a vaccine need against Pf is quite urgent. The life cycle of the malarial organism is divided into the asexual or endogenous cycle in the human and the sexual or exogenous cycle in the mosquito, in this study, it is aimed to develop a functional vaccine against malaria by recombinantly producing the surface protein which plays a key role in the sexual cycle.

The most important point in the development of recombinant proteins is the ability to produce functional proteins. For this, several organisms are used as host organism, such as bacterium, plant, and yeast ex. In this study the transient plant expression system was used for recombinant protein production. The transient system has many advantages over other methods such as ease of manipulation, high yield, speed of production and low cost. And the transient plant expression system advantages are among other things mentioned above, free of bacterial endotoxins, free of over glycosylated proteins by yeast, higher eukaryotes which have post translational modifications like mammalians and lack of animal and human pathogens. All these features provide the optimal conditions for our success in our work.

In this study, the target genes were produced in the plant by agroinfiltration to produce the deglycosylated form of Pfs48/45 (a transmission blocking target protein which present surface of macrogametes of *Plasmodium falciparum*, a potent vaccine candidate against malaria, and the correct folded functional protein was produced.

2. THEORETICAL INFORMATION AND RESOURCES

2.1. Malaria

Malaria has been known for thousands of years in human history and is one of the deadliest illnesses of humanity. There is records of malaria in a Chinese document from 2700 BC, clay tablets from Mesopotamia from 2000 BC, Egyptian papyri from 1570 BC and Hindu texts from 600 BC (Cox 2010).

For thousands of years, people of the Neolithic period, the early Chinese population, the ancient Greeks, the princes and the poor people have been victims of malaria (Arrow KJ, Panosian C, Gelband H 2004). Malaria has taken between 150 and 300 million lives, even in the 20th century alone (Carter and Mendis 2002).

Today, 40 percent of the world's population is living in areas where malaria is contagious, especially in Africa, Amazon, and most of the Asian countries (Arrow KJ, Panosian C, Gelband H 2004)

For 2500 years, the idea that malaria fevers were caused by miasmas from swamps maintained and the word malaria comes from the Italian mal'aria meaning polluted   air.
By the discovery of parasites in the blood of malaria patients by Alphonse Laveran in 1880 the cause of malaria began to be understood. Ronald Ross discovered the transmission in 1897 and in 1898 by the Italian malariologists demonstrated human malaria was transmitted by mosquitoes Anophelines (Cox 2010).

The five human *Plasmodium* species transmitted from person to person are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi*.

The first indication of malaria symptoms resembles simple viral infections. It starts with a headache, fatigue, muscle and joint pain, followed by high fever, perspiration, chills and vomiting. At this stage of disease if the effective antimalarial drugs are given full recovery is expected, otherwise particularly in *Plasmodium falciparum* malaria, lethal severe malaria could be developed. This development may take days or few hours. Severe malaria displays cerebral malaria, acidosis, anaemia, hypoglycaemia, renal failure or pulmonary oedema. If severe malaria left untreated will be fatal (World Health Organization; 2015).

When the infected female anopheline takes a blood meal from a healthy person, injects sporozoites from her salivary glands into the bloodstream. After 30-60 minutes, a preerythrocytic phase which occurs within hepatic cells in the liver, antedates bloodstream invasion by the parasite. Before attacking the liver cells, they engage in cell receptor via circumsporozoite protein. Over the next 5 to 25 days, the sporozoite reproduces by asexual binary fission and create liver schizont. During this course, there is no symptom in the patient. At the end of one week, the schizonts rupture the liver cells and free merozoites into the bloodstream, parasites grow and transform to trophozoites and become blood schizont. The erythrocytic cycle, the most important pathogenetically, begins with the merozoite invasion of red blood cells. Whereas *P. vivax*

and *P. ovale* invade only reticulocytes, *P. falciparum* merozoites can invade any erythrocyte. The merozoites become trophozoites, which in turn undergo either shizogony or gametogony within the erythrocyte. When the erythrocyte ruptures, the merozoites liberated will parasitize other red blood cells. The debris of the ruptured cells, together with the merozoites and their metabolic by-products, is set free in the bloodstream and act as a pyrogen. Once this happens, symptoms such as fever, sweating and shivering begin to appear. If enough *P. falciparum* parasites pass into the brain vessels, cerebral malaria will occur (Arrow KJ, Panosian C, Gelband H 2004)

Some of the trophozoites within the red blood cells become differentiated into round or crescentic sexual forms called microgametocytes and macrogametocytes. These gametocytes are taken up by the *Anopheles* mosquito and undergo maturation in 7 to 12 days. The male microgamete then fertilizes the female macrogamete, producing a fertilized cell or zygote. The zygote penetrates the Wall of the stomach and forms an oocyst. Large number of sporozoites develop within this cyst. The oocyst ruptures, and the sporozoites then reach the body cavity of the mosquito. From the body cavity the sporozoites invade other parts of the mosquito's body. Those reaching the salivary glands pass down the proboscis when the insect bites, thus infecting a human.

For prophylaxis, several drugs are recommended for different area and must be combined with personal protective measures. The drugs are not 100% protective; the person who travel the malaria transmission areas must use insect repellent and an insecticide-treated bed net as long as they stay in area. Atovaquone/Proguanil, Chloroquine, Doxycycline, Mefloquine, Primaquine drugs are used for prophylaxis, However, these drugs should be preferred according to the drug resistance in the area, the health status of the person, pregnancy and breastfeeding, and whether they are pediatric tablets for children. Some of these drugs need to be started weeks prior to travel and some of them taking daily or weekly. The side effects or drug-drug interactions can be seen. For prophylaxy, a vaccine for malaria urgently needed (Anonymous 2).

Treatment of malaria depends of which *Plasmodium* species cause the infection. For treatment a different drug from chemoprophylaxis should be used. Chloroquine, atovaquone-proguanil, artemether-lumefantrine, mefloquine, quinine, quinidine, doxycycline combined with quinine, clindamycin combined with quinine, artesunate can be used for treatment. How to choose a drug for treatment depends on the species of parasite, the area acquired of infection, drug resistance condition, clinical situation of patient, pregnancy, drug allergies and other drugs taken by the patient. Most of the drugs are used for blood schizonts (Anonymous 3).

2.2. *Anopheles*

Anopheles taxonomy lineage is:

Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Protostomia; Ecdysozoa; Pa narthropoda; Arthropoda; Mandibulata; Pancrustacea; Hexapoda; Insecta; Dicondylia ; Pterygota; Neoptera; Holometabola; Diptera; Nematocera; Culicomorpha; Culicoide a; Culicidae; Anophelinae; Anopheles; Cellia; Pyretophorus; gambiae species complex

Anopheles is a genus of mosquitos which contain about 465 species and is the most studied genus of mosquitos because of the great impact on human health. 60 species of it are malaria vectors and 30 of them have the major role (Arrow KJ, Panosian C, Gelband H 2004).

Especially *Anopheles gambiae* is responsible of most of the malaria transmission because of its long life compared other species of Anopheles and preferred to feed on humans. It's carries *Plasmodium falciparum* in most of the Africa areas and cause severe malaria. Most of the species of Anopheles live in tropical regions but some species survive altered temperatures and even in Arctic summer (Bruce-Chwatt 1985).

Only the female anopheline feeding on human and transmit the parasite that caused malaria. After receiving a blood meal, the female anopheline will find a protected resting area and while digesting the blood will wait until the eggs are fully developed. When ready, she will leave her eggs to a source of water and fly to investigate another blood meal. Adult female leaves about 50 to 200 eggs at a time. Developments take place at four different stages - eggs, larvae, pupae and adults. When eggs, larvae and pupae streaks occur on the surface of the clean water and just below the surface of the water, adults can go to different places with the ability to fly in a few minutes. Anopheles are laid separately and float on the surface of the water. Eggs can hatch in 2-3 days after they are laid, but they can last up to three weeks in cold climates. The larvae take air from the body's special organs and must come to the surface of the water to breathe. The larvae feed on bacterium, algae and other microorganisms in the water and undergo four larval stages. At the end of which the pupa develops and completes this phase by breathing with a pair of organs called respiratory trumpets for 2-3 days on the surface of the water. Adult anophelines emerges from the pupae and live on average 2-3 weeks (Anonymous 4).

Figure 2.1. Anopheles sp.   (Anonymous 5)
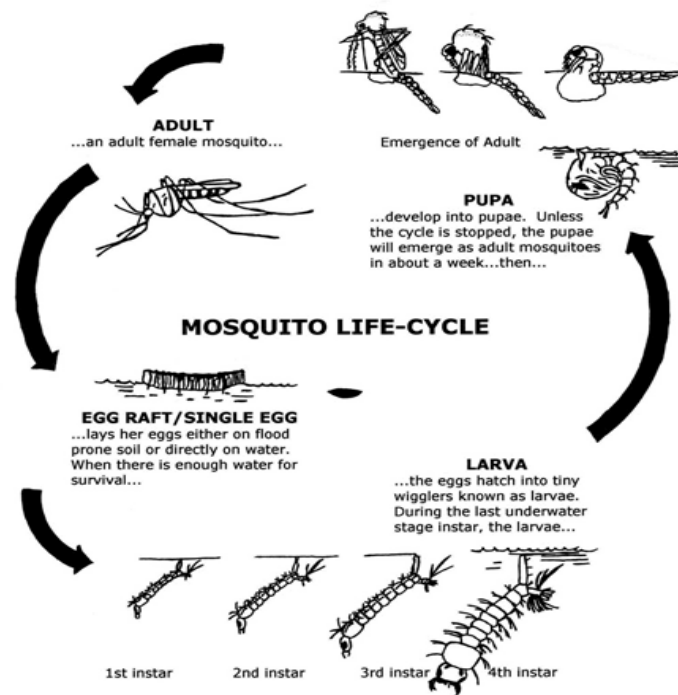
Figure 2.2. *Anopheles sp.* mosquito life cycle (Anonymous 6)

2.3. *Plasmodium falciparum*

Plasmodium falciparum taxonomy lineage is: *Eukaryota; Alveolata; Apicomplexa; Aconoidasida; Haemosporida; Plasmodiidae; Plasmodium; Plasmodium (Laverania) falciparum*

Plasmodium falciparum differs from other *Plasmodium* species by the severity of the caused disease called falciparum malaria. The infection in falciparum malaria is asymptomatic, a nonimmune host could develop fatal symptoms such as cerebral malaria, severe anemia and organ failure. The deadliest malaria type is the falciparum malaria, not just because of the prognosis to severe malaria, also size of the area and the number of the population of get infected. A total of 2.57 billion people in 85 countries are living in the area where falciparum malaria is endemic and are at risk of infection (Rossati et al. 2016).

There is a direct proportionate relationship between malaria and poverty. Malaria endemic countries have lower economic growth rates. While continuing to have negative impacts on issues such as fertility, population growth, work and worker productivity, it leads to major economic problems with an increase in early death rate and medical costs (Sachs and Malaney 2002).

Resistance to drugs used against *Plasmodium falciparum* has adversely affected the war against falciparum malaria. New anti-malarial drug combination are used but this is not the solution, a holistic approach is needed for the solution (Thu et al. 2017).

Figure 2.3. *Plasmodium falciparum* illustration draw (Anonymous 7)

Figure 2.4. Electron microscopy image of *Plasmodium falciparum* sporozoites (Anonymous 8).

Figure 2.5. *Plasmodium sp.* life cycle (S Griffith et al. 2007)

2.4. Vaccine Against Malaria

Malaria is one of the deadliest disease in human history. *Plasmodium falciparum* caused type malaria is the most fatal type and there is no immunity against it. The parasite has passed through a unique evolutionary process to escape the immune system. Despite 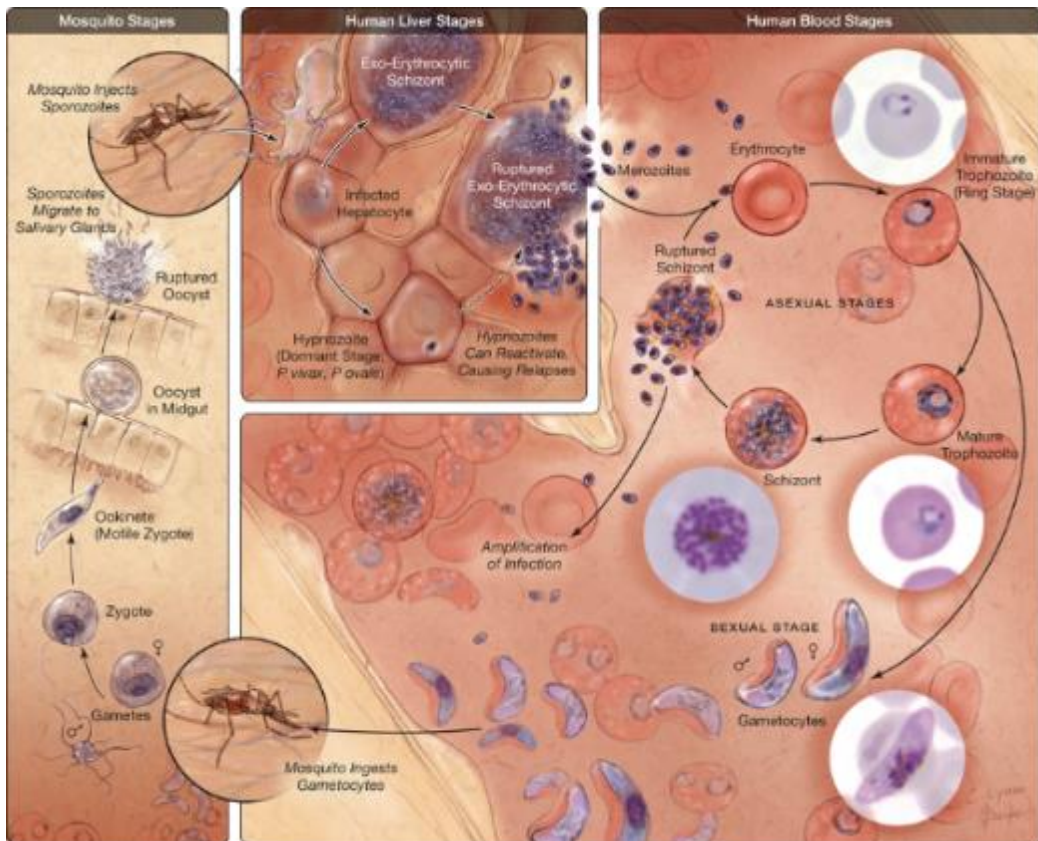the presence of many barriers to fight parasites in the immune systems of humans and mosquito hosts, they have been able to transmission and infection thanks to different life cycles (Gomes et al. 2016). Because of these reasons, despite the work for decades, a vaccine has not been developed yet. Two main research flows have been developed for vaccine trials. One is the development of subunit vaccines from well-defined parasite antigens. The other line of research is the development of a vaccine based on the use of all attenuated sporozoites (Long and Zavala 2016). Pfs48/45 shows conformational epitopes and is a candidate for vaccine for malaria transfer-blocking (TB) immunity. Monoclonal antibodies (mAbs) against Pfs48/45 prevent fertilization (Outchkourov et al. 2007). A multi-stage vaccine could be the next step toward malaria elimination and eradication (Tran et al. 2015). There is another approach to produce a vaccine against malaria targeting the sporozoites. However, immunization with this method has been considered to be impractical and crude for large applications (Patarroyo et al. 1990)

2.5. Glycosylation

Glycobiology is the study of the structure, biosynthesis and evolution of glycans. N-glycan synthesis in eukaryotes is partially preserved in endoplasmic reticulum although n-glycan and O-glycan biosynthesis in golgi body is highly specific and results in different oligosaccharide structure in plants and mammals due to glycoproteins (Gomord et al. 2010). The use of plants as alternative to mammalian cell lines to produce therapeutic glycoproteins requires optimization of the protein N-glycosylation in the plant to suit the plant. Most of the eukaryotic proteins considered to be biopharmaceuticals in the Swiss-Prot database are 50% glycoproteins (Walsh and Jefferis 2006). The binding of carbohydrates to proteins is classified into two different categories. N-glycans bind to the amide group of asparagine (ASN) in the protein chain, and O-glycans bind to the serin (Ser) treonine (thr) hydroxylase or hydroxyproline (Hyp) residues.

N-glycosylation occurs in endoplasmic reticulum (er), where lipid-bound oligosaccharides transfer to newly formed polypeptides after Co–translation or translation. O-glycans are synthesized in endoplasmic reticulum (er) and golgi body during the transfer of monosaccharides to folded proteins (Gomord et al. 2010). The biggest obstacle to the use of phyto-produced pharmaceuticals in the treatment of human diseases is the inability of plants to phosphorylate human species in biopharmaceuticals. Protein N-glycolation in plant cells begins with the transfer of the oligosaccharide precursor, (Glc3Man9GlcNAc2) to the specific Asn residue of the N-glycosylation sequence (Asn-X-Ser / Thr) protected from the dolichol lipid transporter. Primarily begins to transfer immature protein. Then transported along the glycoprotein secretory pathway and N-glycan is exposed to some maturation processes involving stages such as removal of glucose and mannose residues to form high-mannose n-glycans. Ultimately, in the endoplasmic reticulum and in the golgi apparatus the addition of new sugars causing the formation of complex N-type glycans occurs. While β 1,2-xylose and α 1,3-fucose found in plants are absent in mammals, β 1,4-

galactoside bound sialic acid present in mammals is not found in plants (Lerouge et al. 1998).
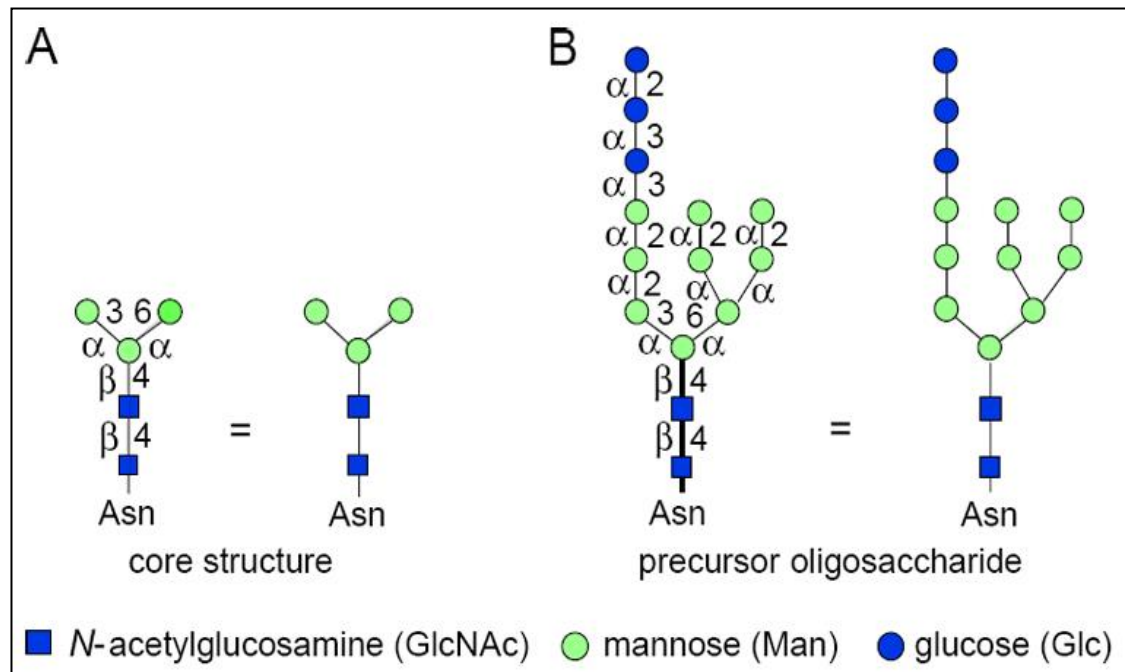

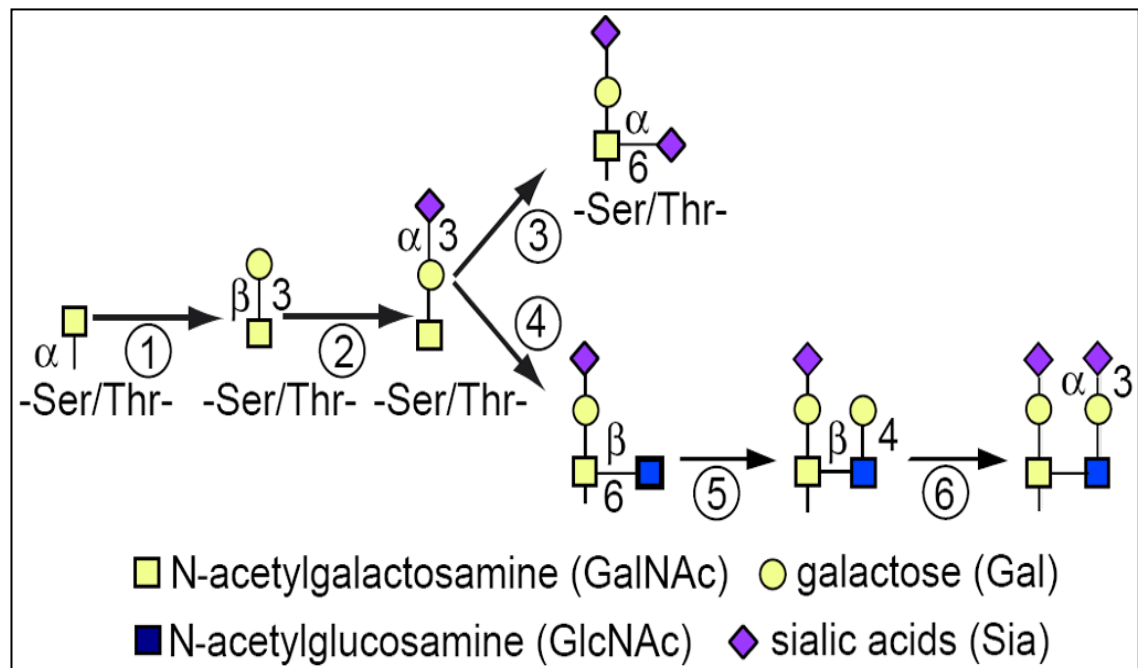**Figure 2.3.** Drawing of the precursor and core region structure of N-linked sugar chains(Freeze and Kranz 2010).

Figure 2.4. small portion of the O-linked glycosylation pathway (Freeze and Kranz 2010).
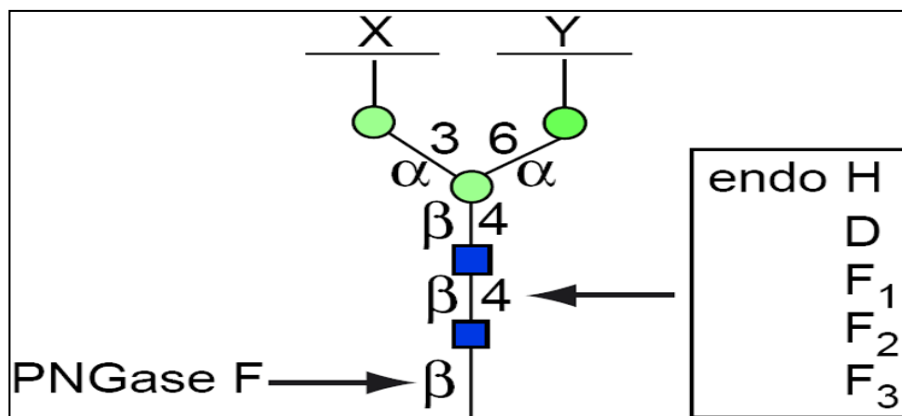
Figure 2.5. Endoglycosidase-sensitive bonds in the nucleus of PNGase F and N-linked glycans. PNGase F intercepts GlcNAc and Asn and converts Asn to Asp. Endoglycosidases (H, D, and F) leave one GlcNAc bound to the protein by interrupting the bond between the two GlcNAc at the core region (Freeze and Kranz 2010).

In both mammalian and plant cells, the glycan processing mechanisms are located in the endoplasmic reticulum (high mannose) and the golgi apparatus (complex glycan). Production of the target protein in the plant takes place in two basic strategies; transgenic and transient gene expression (Yusibov and Mammedov 2010). In the transgenic system, the target gene is joined to the plant nuclear genome or the chloroplast genome. In the transient gene expression system, the genetically engineered plant viruses carrying the target gene are included in the plant, and the recombinant protein becomes expressive without being included in the plant host genome (Franken, Teuschel, and Hain 1997; Daniell, Streatfield, and Wycoff 2001). Transient gene expression has several advantages over stable transformation; time-efficient, the target protein has high expression, the system is scalable and stable, it has the ability to produce with less environmental damage (Yusibov et al. 2006; Roy et al. 2010).

Generally, most therapeutic proteins require at least proteolytic cleavage and glycolysis for their bioactivity, pharmacokinetics, stability and solubility. Most plant proteins are synthesized as preproteins in the secretory pathways. In plant cells as well as in other eukaryotic cells, proteins are directed to the secretory pathway by the amino grup-terminal signal peptide. After the signal peptide are leave, the proteins are release into the ER lümen. The protein cannot fold or combine correctly. Most plant proteins leave the ER lumen as proprotein, and the proprotein contains polypeptides that will be lost during proteolytic maturation in the protein secretory pathway. In most of the therapeutic proteins, sugar is linked to the amide nitrogen of the asparagine (N-glycosylation) or the hydroxyl end (O-glycolysis) of the threonine or serine in the peptide chain. Binding of carbohydrates to polypeptide chain strongly affects the physicochemical properties of the protein, such as resistance to thermal denaturation, protection from proteolytic degradation, and solubility (Gomord and Faye 2004). At the same time, the protein may alter important biological functions such as immunogenicity, specific activity and ligand-receptor interactions. Asparagine N-linked oligosaccharides are exposed to some protein maturation reactions involving sugar addition or truncation processes in the ER and Golgi apparatus while being transported along the glycoprotein secretory pathway. The main difference in plant and mammalian N-glycine ripening; in mammals, α (1,6) -containing

fucose residues and terminal sialic acid are formed in the golgi apparatus; plants have β (1,2) xylose and α (1,3) fucose branching. Different strategies can be applied during the design of N-glycolysis in plants. The most plausible strategy is to prevent the addition of immunogenic glycans to the phytochemicals produced. For this, the protein should be stored in the ER, meaning should be preventing the passage to the Golgi cisterna where the immunogenic glycopeptides are added. For this reason, the KDEL sequence is added to the carboxy terminal of the protein. In this way, the addition of high mannose N-glycans to the protein, which would reduce the immunogenic activity of the protein, would be prevented. Another important strategy is the inhibition of Golgi glycosyltransferases. The silencing of the genes of α (1,3) fucosyltransferase and β (1,2) xylosyltransferases allows the production of protein without plant-specific glycoepithelium (Koprinovova, A., Lienhart, O., Decker, E.L., Stemmer, C., Wagner, S., Gorr 2003). Another attractive strategy is to design plant N-glycans in plants to express mammalian glycosyltransferases. The biological activity of most therapeutic glycoproteins, such as antibodies, blood proteins and interferons, is dependent on their glycosylation state and explains how these biopharmaceuticals are produced along with their glycosylation ability in the heterologous expression system (Balen and Krsnik-Rasol 2007).

2.6. Aberrant Glycosylation and *In vivo* Deglycosylation Strategy

Plant based transient expression system is a promising technology to produce various recombinant proteins including vaccine antigens, therapeutic proteins, antibodies and industrial enzymes. These systems offer superior benefits over other expression systems, which include: quick production timeline, low cost input, highly scalable, high production capacity and they do not harbor mammalian pathogens. In addition, because plants have eukaryotic post-translational modifications, including N-linked glycosylation, the technique may be particularly useful for the expression of glycosylated proteins including mammalian proteins, in which N-glycosylation is critical for their functional activity. However, the ability of plants to glycosylate proteins also can be a significant limitation on the usefulness of plant-based expression systems. Some proteins (e.g., Plasmodium parasites proteins, PA83 of Bacillius Anthracis etc.,) lack machinery for N-linked glycosylation and proteins native to such species may contain multiple potential glycosylation sites that could be aberrantly glycosylated when expressed in plants, leading to reduced functionality and immunogenicity due to incorrect/altered folding or masking of epitopes. To solve this problem a strategy was recently developed by in *vivo* deglycosylation of recombinant proteins with bacterial PNGase F (Mamedov et al, 2012; Mamedov et al., 2016) and Endo-β-N-acetylglucosaminidase (EC3.2.1.96, Endo H) in plants (Mamedov et al., 2017). Plant produced PNGase F deglycosylated PA83 was found to be more stable and shows significantly higher levels of toxin-neutralizing antibody titers in immunized mice compared to its glycosylated plant produced PA83 counterpart (Mamedov et al., 2016). Quite recently, the work performed in our laboratory was demonstrated that enzymatic deglycosylation of target proteins *in vivo* by Endo H had a positive impact on the stability and the deglycosylated PA83 molecule produced by Endo H appeared to be more stable than the PNGase F deglycosylated counterpart, which may greatly increase the life and duration of vaccine storage and thereby reduce the cost of the vaccine considerably. Thus, Endo H deglycosylated plant produced PA83 of *B. anthracis* are expected to be most advanced

vaccine candidate against anthrax. Taken together, the data supports the fact that the Endo H co-expression strategy provides another opportunity to produce important vaccine antigens, including a vaccine against anthrax, TB vaccines against malaria, and therapeutic proteins, antibodies, and recombinant enzymes for therapeutic use and industrial applications. In summary, this and our previous studies collectively demonstrate that enzymatic deglycosylation of target proteins *in vivo* has the potential to become a robust strategy to produce non-glycosylated proteins in plants.

3. MATERIALS AND METHODS

3.1. Cloning The Genes

The full length of Pfs48/45 were optimized for expression in Nicotiana benthamiana and synthesized by GENEART AG. PR-1a signal peptide (MGFVLFSQLPSFLLVSTLLLFLVISHSCRA) was added to the N-terminus and the KDEL sequence (the ER retention signal) and the FLAG epitope (the affinity purification tag) were added to the C-terminus. The material was amplified by PCR using F and R primers which are:

F=LP16CF1(58,4℃) : GAGTCaccggtATGGGTTTCGTGCTGTTCAGC

R=LP16CR1(60,3℃): ATGAGGCCCAGctcgagC

For PCR method, 10µL 5x Phusion Buffer, 0,5µL Phusion DNA Polymerase (Phusion Green High-Fidelity DNA Polymerase, Thermo Scientific, Catalog Number: F-534S), 1µL 10mM dNTP, 2,5µL 10mM F primer, 2,5µL 10mM R primer, 1µL Template DNA, and 32,5µL DNAase / RNAase / Protease-free water placed in a PCR tube and mixed gently and placed in PCR device. Annealing temperature calculated as: (54,4+60,3)/2-5=54℃ and the PCR process carried on according to manufacturer's protocol.

When the PCR process is done, the verification has been made by electrophoresis. For this, 5µL sample taken from 50µL PCR product and mixed gently in an Eppendorf tube with 5µL ddH$_2$O and 2µL Dye (Thermo Scientific 6x, Catalog No: R0611) and were run on a 1% agarose gel in 1xTAE buffer solution with 3µL GeneRuler 1kb DNA Ladder (Thermo Scientific) in 110V. for 30mn. and gel separation was observed. After verification, the PCR product cleaned by DNA Clean & Concentrator (Zymo Research, Catalog Number: D4006) according to manufacturer's protocol. The PCR product digested with Age1 and XhO1 enzymes and verified by electrophoresis, rest of them cleaned by DNA Clean & Concentrator (Zymo Research, Catalog Number: D4006) according to manufacturer's protocol.

The pEAQ binary expression vector (Sainsbury, Thuenemann, and Lomonossoff 2009) (provided by Dr. George P. Lomonossoff, John Innes Center, Biological Chemistry Department) loaded to 1% agarose gel in 1xTAE buffer solution with 3µL GeneRuler 1kb DNA Ladder (Thermo Scientific) and run on 110V. for 35 minute. Under U.V. light, the band was cut with help of a lancet and recovered from the gel according to manufacturer's (Zymoclean Gel DNA Recovery Kit, Catalog No: D4007) protocol. After recovery, 9µL of DNAase / RNAase / Protease-free water and 2µL of loading dye (Thermo Scientific 6x, Catalog No. R0611) are added to 1µL sample and loaded to 1% agarose gel in 1xTAE buffer solution with 3 µL GeneRuler 1kb DNA Ladder (Thermo Scientific) and ran on 110V. for 40 minutes for verification. When the verification is done, modified Pfs48/45 and the pEAQ binary expression vector ligated using Quick Ligation Kit (New England BioLabs, Catalog Number: 1091410) according to manufacturer's protocol.

pGreen vector (Hellens et al. 2000) digested with XhO1 and Pac1 enzymes at 37°C for an hour and loaded to 1% agarose gel in 1xTAE buffer solution with 4μL GeneRuler 1kb DNA Ladder (Thermo Scientific) and run on 120V. for 40 minutes. Under U.V. light, the band was cut with help of a lancet and recovered from gel according to manufacturer's (Zymoclean Gel DNA Recovery Kit, Catalog No: D4007) protocol. After recovery, 9μL of DNAase / RNAase / Protease-free water and 2μL of loading dye (Thermo Scientific 6x, Catalog No. R0611) are added to 1μL sample and loaded to 1% agarose gel in 1xTAE buffer solution with 3 μL GeneRuler 1kb DNA Ladder (Thermo Scientific) and ran on 110V. for 30 minutes for verification.

The Endo H gene (GenBank accession AAA26738.1) was optimized and was purchased after being synthesized by GENEART AG (Thermo Fisher Scientific).

To express Endo H in *N. benthamiana* plants, the signal peptide was replaced with the *Nicotiana tabacum* PR-1a signal peptide (MGFVLFSQLPSFLLVSTLLLFLVISHSCRA). In addition, KDEL were added to the C-terminus.

The PNGase F gene was also co-expressed with target proteins. The sequence of PNGase F (GenBank accession number: J05411) was optimized and purchased after being synthesized at Integrated DNA Technologies (IDT) with KDEL (a C-terminal ER retention peptide). Non-tagged versions of the Endo H and PNGase F genes, with KDEL but lacking the FLAG epitope, were constructed to avoid co-elution of plant produced recombinant Endo H or PNGase F with FLAG- tagged target proteins (Mamedov et al. 2017).

The generated genes were added to pGreen vector to obtain pGr-Endo H and pGr-PNGase F (Hellens et al. 2000)

The modified Endo H and modified PNGase F genes also digested with XhO1 and Pac1 enzymes at 37°C for an hour and loaded to 1% agarose gel in 1xTAE buffer solution with 5μL GeneRuler 1kb DNA Ladder (Thermo Scientific) and run on 120V. for 60 minutes. Under U.V. light, the band was cut with help of a lancet and recovered from gel according to manufacturer's (Zymoclean Gel DNA Recovery Kit, Catalog No: D4007) protocol. After recovery, 9μL of DNAase / RNAase / Protease-free water and 2μL of loading dye (Thermo Scientific 6x, Catalog No. R0611) are added to 1μL sample and loaded to 1% agarose gel in 1xTAE buffer solution with 3 μL GeneRuler 1kb DNA Ladder (Thermo Scientific) and ran on 110V. for 45 minutes for verification. When the verification is done, modified Endo H gene and the pGr vector and modified PNGase F gene and the pGr vector ligated using Quick Ligation Kit (New England BioLabs, Catalog Number: 1091410) according to manufacturer's protocol.

3.2. Transfer to *E. coli* XL1Blue Competent Cells

Ligated sample (pEAQ-Pfs48/45-16) incubated in room temperature for 20 minutes and then transferred to *E. coli* XL1Blue competent cell by heat-shock method. According to this method, the ligation product (21 μl) was added onto *E. coli* XL1Blue competent cells (100 μl) and incubated for 5 min. on ice. Subsequently, the mixture was left in the water bath at 42 ° C for 50 seconds and immediately transferred to ice container

and kept on ice for 5 minutes. On the samples, 400 µl of LB broth was added and incubated at 37 ° C for 1 hour at 225 rpm. The resulting culture was centrifuged at 2000xg for 3 min. to allow cells to settle. Subsequently, 100 µL of the supernatant was added to the collapsed cells, and the collapsed cells were resuspended by pipetting, and spread to the LB plate containing 50 µg / ml kanamycin by pre-sterilized glass spreader and left overnight at 37 ° C in an incubator. After overnight incubation, colonies observed on petri dish. The ligated sample contained colonies incubated overnight at 225 rpm at 37 ° C in 5 ml of liquid LB broth containing 50 µg / ml kanamycin. Plasmids in *E. coli* XL1Blue cells were isolated according to the manufacturer's recommendations with the Zyppy Plasmid Miniprep Kit (Zymo Research, USA, Catalog Number: D4037). Isolated plasmids digested with XhO1 and Age1 enzymes for 1 hour at 37°C and verified by electrophoresis (1% agarose gel in 1xTAE buffer solution with 3µL GeneRuler 1kb DNA Ladder (Thermo Scientific) and run on 110V. for 55 minute). A portion of the *E. coli* XL1Blue cells contained plasmids was stocked with 90% glycerol and stored at -80 ° C.

Ligated samples (pGr-Endo H and pGr-PNGase F) incubated in room temperature for 20 minutes and then transferred to *E. coli* XL1Blue competent cell by heat-shock method. According to this method, each ligation product (21 µl) was added onto *E. coli* XL1Blue competent cells (100 µl) and incubated for 5 min. on ice. Subsequently, the mixture was left in the water bath at 42 ° C for 50 seconds and immediately transferred to ice container and kept on ice for 5 minutes. On the samples, 400 µl of LB broth was added and incubated at 37 ° C for 1 hour at 225 rpm. The resulting culture was centrifuged at 3000xg for 2 min. to allow cells to settle. Subsequently, 100 µL of the supernatant was added to the collapsed cells, and the collapsed cells were resuspended by pipetting, and spread to the LB plate containing 50 µg / ml kanamycin by pre-sterilized glass spreader and left overnight at 37 ° C in an incubator. After overnight incubation, colonies observed on petri dish. The ligated samples contained colonies (pGr-Endo H and pGr-PNGase F) incubated overnight at 225 rpm at 37 ° C in 5 ml of liquid LB broth containing 50 µg / ml kanamycin. Plasmids in *E. coli* XL1Blue cells (pGr-Endo H and pGr-PNGase F) were isolated according to the manufacturer's recommendations with the Zyppy Plasmid Miniprep Kit (Zymo Research, USA, Catalog Number: D4037). Isolated plasmids (pGr-Endo H and pGr-PNGase F) digested with XhO1 and Pac1 enzymes for 1 hour at 37°C and verified by electrophoresis (1% agarose gel in 1xTAE buffer solution with 3µL GeneRuler 1kb DNA Ladder (Thermo Scientific) and run on 120V.   for   30   minute). A portion of the *E. coli* XL1Blue cells contained plasmids (pGr-Endo H and pGr-PNGase F) was stocked with 90% glycerol and stored at -80 ° C.

3.3. Transfer to *Agrobacterium tumefaciens* AGL₁Psoup Strain Competent Cells

After verification, plasmids isolated from *E. coli* XL1 Blue cells (pEAQ-Pfs48/45-16) were transferred into *Agrobacterium tumefaciens* AGL₁psoup strain competent cells by electroporation with electric shock (1µL DNA+100µL AGL₁psoup strain competent cells, 2470V. 10,3m/s).After adding 1 ml. SOC Medium to electroporation cuvette and gently pipetted, cells left for incubation for 2 hours. These bacteria were then spread on the LB plate containing 50 µg / ml kanamycin by pre-sterilized glass spreader and allowed to incubate at 28 ° C non-shaker incubator for

overnight. After 3 days the colonies were observed. The appropriate colonies were selected and transferred to a BBL medium containing kanamycin and left to shake at 225 rpm at 28 ° C for overnight incubation. After approximately 24 hours, the bacteria that have been grown are O.D. (Optical Density) is measured (O.D. should be ≥ 1). A portion of the bacterial solution was removed, stocked with 90% glycerol and stored at -80 ° C.

After verification, plasmids isolated from *E. coli* XL1 Blue cells (pGr-Endo H and pGr-PNGase F) were transferred into *Agrobacterium tumefaciens* AGL$_1$psoup strain competent cells by electroporation with electric shock (1µL DNA+100µL AGL$_1$psoup strain competent cells, 1495V. 5,3m/s for pGr-Endo H and 1498V. 6,7m/s for pGr-PNGase F).After adding 1 ml. SOC Medium to electroporation cuvette and gently pipetted, cells left for incubation for 2 hours. These bacteria were then spread on the LB plate containing 50 µg / ml kanamycin by pre-sterilized glass spreader and allowed to incubate at 28 ° C non-shaker incubator for overnight. After 3 days the colonies were observed. The appropriate colonies were selected and transferred to a BBL medium containing kanamycin and left to shake at 225 rpm at 28 ° C for overnight incubation. After approximately 24 hours, the bacteria that have been grown are O.D. (Optical Density) is measured (O.D. should be ≥ 1). A portion of the bacterial solution was removed, stocked with 90% glycerol and stored at -80 ° C.

3.4. Infiltration To *Nicotiana benthamiana* Plants

After the O.D values were measured and recorded, the bacteria (AGL$_1$psoup pGr-Endo H , AGL$_1$psoup pGr-PNGase F and AGL$_1$psoup pEAQ-Pfs48/45-16C) were centrifuged at 5000xg for 5 min., the supernatant was emptied, and the pellet were placed in the MMA medium supplemented with 100 mM acetosyringone (according to O.D., 15µL acetosyringone for 100ml MMA Medium). The bacteria were then mixed in the magnetic stirrer for 2 hours at room temperature. The bacteria are now ready for plant infiltration. For infiltration, plants that reached a certain size of 6-7 weeks old from sowing of seeds were used. The bacterial solution was injected into the plant leaves using a syringe with co expression AGL$_1$psoup pEAQ-Pfs48/45-16C /pGr-Endo H and AGL$_1$psoup pEAQ-Pfs48/45-16C /pGr-PNGase F constructs. Plant infiltration was optimized with the use of different ratio (pEAQ-Pfs48/45-16C (0,9)/ pGr-Endo H (0,1) ratio and pEAQ-Pfs48/45-16C (0,5)/ pGr-PNGase F (0,5)).

Figure 3.1. *Nicotiana benthamiana* growing from seeds and infiltration process.

3.5. SDS-Page, Native-Page and Western Blot Analysis

Infiltrated leaves were collected at 7 dpi and homogenized using mortar and pestle in three volumes of extraction buffer. Homogenized samples centrifugated at 13000xg for 5 minutes and supernatant diluted two-fold in the SDS sample buffer. SDS-PAGE was applied in 10% acrylamide gels with 10µL samples and then stained with Coomassie bright blue dye. For immunoblot analysis, polyvinylidene fluoride membranes (Millipore, Billerica, MA) were blasted for 1 hour at 100 V using transfer buffer after running the samples at first 100V, 13 min. and then 200V, 45 min. in 10% acrylamide gel using running buffer. Membranes were first blocked by shaking in 5 min. 0.5% I-Block (Applied Biosystems, Carlsbad, Calif.) To allow buffer solutions to be removed and then blocked for 1 hour at room temperature with 0.5% I-Block reactive 1xTBS (Tris Buffered Saline). Membranes were labeled at 1 hour at room temperature with purified anti-DYKDDDDK tag antibody (anti-FLAG antibody) (BioLegend, Cat. No. 637301) added to I-Block-reacted 1xTBS. After labeling, the membranes were washed 3 times for 5 min. with 0.5% I-Block solution (Applied Biosystems, Carlsbad, Calif.) and 1x TBS for 5 min. and then labeled with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Cat. No. ab97051, Abcam).

Reducing samples were prepared in 5X Laemli Buffer with SDS included. Non-reducing gel and samples (native samples) were prepared in the same way with no SDS and reducing agent added. And the Native PAGE is performed using native samples and running buffers without denaturants or SDS and proteins were explored using anti-Flag antibodies.

MRA-26, mouse hybridoma cells producing mAb anti-Pfs48/45 was obtained from Malaria Research and Reference Reagent Resource Center (MR4). Prior to inoculating the cells, the frozen flask was immediately dissolved in the water bath at 37 ° C. Before opening, flask was wiped with 70% ethanol. Cells were resuspended in RPMI

medium. Then centrifuged at 2700 g for 5 minutes at 37 ° C in a 15 ml falcon tube. Supernatant was discarded, and cells were dissolved in the same manner as explained above. Totally 12 ml medium and cells in the T25 flask were placed vertically in the incubator. Cells were passaged in 1:5 ratio in every three days. Hybridoma cells were cultured for about two weeks. During the last passage, to obtain a final FBS concentration of 2%, RPMI medium without serum was added on serum-grown cells. Reduced serum medium was isolated from cells with a rapid spin and filter sterilized through 0.4-micron membrane. Almost 500 ml filter sterilized medium and 5x106 cells were kept in -80˚C as apart. Anti-Pfs48/45 mAb was purified from the medium using Protein A affinity column.

Finally, chemiluminescence detection was performed with SuperSignal West Pico Stable Peroxide (2.5 mL) and Luminol / Enhancer (2.5 mL) solutions (SuperSignal West Pico, Thermo Fisher Scientific Grand Island, NY), ECL Western blotting reagents. Photographs were taken using a GeneGnome XRQ chemiluminescence imaging system (Syngene Corp., USA). Protein quantities were mathematically calculated using GeneTools Software.

3.6. Purification

Pfs48/45 was designed and ex- pressed with a C-terminal FLAG epitope. Purification of plant produced Pfs48/45 variants (glycosylated and deglycosylated) were performed using Anti-DYKDDDDK Affinity Gel (Cat. No. 651503, BioLegend) according to manufacturer's protocol. Before the purification process, the buffers for Flag-tag purification was prepared and calculated necessary amounts for each sample. For purification of plant produced glycosylated and non-glycosylated Pfs48/45 variants from *N. benthamiana*, 10 g of frozen leaves from each sample was ground in three volumes of extraction buffer (1mM DIECA+30ml Equilibration buffer) on ice using mortar and a pestle. Plant debris was removed by filtration through miracloth followed by centrifugation at 20,000 g for 10 minutes and then filtered through a 0.45μm syringe filter (Millipore). According to the manufacturer's instructions, affinity column prepared with 0,75ml resin with equilibration buffer and the column was washed with ten volumes of resin equilibrated with buffer. By loading the supernatant to column, bound proteins were eluted using elution buffer (200 mM Glycine, 150 mM NaCl, pH 2.2) into tubes containing neutralization buffer (2.0M Tris pH:8.0) to neutralize. Eluted bound proteins were analyzed by SDS-PAGE and after combined, concentrated with a 30k MWCO Amicon Ultra 0,5ml Concentrator. Concentrated samples were analyzed by SDS-PAGE and stored -80°C until use.

3.7. Solutions And Buffers

- **1% Agorose gel:**

Weigh 0.6 g of agarose and place in 60 ml of 1x TAE buffer. The agarose is allowed to stand in the microwave for about 1-2 min. until completely dissolved. After the temperature of the solution drops, add 1.2 μL of Ethidium Bromide (EtBr). The mixture is poured into a pre-prepared horizontal electrophoresis tank without blistering and the appropriate comb is placed. Gelatin is expected to solidify.

- **50xTAE buffer solution:**

242G. Tris was dissolved in autoclaved 700 mL ddH$_2$O. The mixture was dissolved by adding 100 mL of 0.5 M EDTA (pH: 8) and 57.1 mL of glacial acetic acid. After the pH was adjusted to 8.5-8.6, the final volume was supplemented to 1000 mL with autoclaved ddH$_2$O.

- **1xTAE buffer solution:**

20 mL of 50xTAE buffer solution was added to 1000 mL of final volume with autoclaved ddH$_2$O.

- **1 M CaCl$_2$:**

1.47G. CaCl$_2$ was weighed and dissolved in 10 mL autoclaved ddH$_2$O

- **0.1 M CaCl$_2$:**

1 mL CaCl$_2$ solution added to 9 mL bidistilled water and dissolved.

- **0.1M CaCl$_2$ / 15% Glycerol:**

1 mL of 1M CaCl$_2$ solution and 3 mL of 50% glycerol, dissolved in 6 mL of autoclaved ddH$_2$O.

- **LB-Broth:**

25 G. LB-Broth dissolved in 1000 mL of purified water. Autoclaved at 121 ° C for 30 min.

- **70% alcohol:**

70 mL of ethyl alcohol was mixed with water as the final volume was 100 mL.

- **I-block:**

0.5 G. I-block was mixed thoroughly for 3 to 5 hours on a magnetic stirrer in 1 × TBS as the final volume was 100 mL.

- **10% SDS:**

10 G. of sodium dodecyl sulfate (SDS) added to 90 mL of autoclaved ddH$_2$O and were allow complete dissolution.

- **1xRunning Buffer:**

3.03 G. Tris, 14.3 G. glycine, 10 mL 10% SDS dissolved in autoclaved ddH$_2$O to a final volume of 1000 mL.

- **1x Native Running Buffer:**

3.03 G. Tris, 14.3 G. glycine dissolved in autoclaved ddH_2O to a final volume of 1000 mL.

- **1x Transfer Buffer:**

5.8 G. Tris, 2.93 G. glycine, 370 µL 10% SDS were dissolved in autoclaved ddH_2O to a final volume of 1000 mL.

- **1x Native Transfer Buffer:**

5.8 G. Tris, 2.93 G. glycine were dissolved in autoclaved ddH_2O to a final volume of 1000 mL.

- **5xTBS:**

(20 mM Tris (pH:5,5), 150 mM NaCl) 12,115 G. Tris, 43,88 G. NaCl dissolved in autoclaved ddH_2O to 800 mL. The pH was adjusted to 7.5 with HCl and the final volume was supplemented with 1000 mL of autoclaved ddH_2O.

- **1 × TBS:**

200 mL of 5 × TBS were taken, and the final volume was supplemented to 1000 mL with autoclaved ddH_2O

- **10% APS:**

60 mG. APS was weighed and dissolved in autoclaved ddH_2O to a final volume of 600 µL.

- **1,5 M Tris-HCl (pH: 8,8):**

92.5 G. Tris was weighed and dissolved in 400 mL autoclaved ddH_2O. After the pH was adjusted to 8.8 with 6N HCl, the final volume was supplemented to 500 mL with autoclaved ddH_2O.

- **0.5 M Tris-HCl (pH: 6.8):**

30 G. Tris was weighed and dissolved in 400 mL autoclaved ddH_2O. After the pH was adjusted to 6.8 with 6N HCl, the final volume was supplemented to 500 mL with autoclaved ddH_2O.

- **10 mL Resolving gel (for four gels):**

9.7 mL of autoclaved ddH_2O, 5 mL of 40% Acrylamide-Bis solution, 5 mL of 1.5 M Tris-HCl, 200 µL of 10% SDS, 10µL TEMED was and 100µL 10% APS.

- **10% Stacking gel (for four gels):**

7.95 mL of autoclaved ddH_2O, 1.25 mL of 40% Acrylamide-Bicycle solution, 3,15 mL of 0,5 M Tris-HCl, 125 µL of 10% SDS, 12,5 µL TEMED and 62.5 µL of 10% APS.

▪ **Primary Antibody (Western Blot 1: 500):**

10 μL antibody was dissolved in 10 mL I-block.

▪ **Secondary Antibody (Western Blot 1: 2500):**

4 μL of antibody was dissolved in 10 mL of I-block.

▪ **0.5M EDTA:**

14,612 G. EDTA was weighed and dissolved in 80 mL of autoclaved ddH_2O. After adjusting to pH:8, the final volume was supplemented to 100 mL with autoclaved ddH_2O.

▪ **1M Tris :**

12.114 G. Tris was weighed and dissolved in 100 mL autoclaved ddH_2O.

▪ **0.01% Bromophenol blue solution:**

100 mG. Bromophenol was weighed and dissolved in 20 mL autoclaved ddH_2O.

• **SDS / Western Gel Loading Solution - (5x) Laemnli Buffer:**

7.5 mL of 25% 2-mercaptoethanol was placed in 50 mL of Falkon. Add 660 μL of 100 mg / 20 mL Bromophenol blue stock solution to the solution and mix. The mixture was adjusted to pH = 6.8 with HCl by the addition of 11.9 mL of glycerol, 9.375 mL of 1 M Tris solution and 3.333 mL of SDS. Then the mixture is aliquoted to tubes as 1mL. The tubes were stored at -20 ° C for use. Protein samples are boiled in 1/4 volume (5x) Laemli Buffer before loading gel.

• **SDS gel staining solution (Coomassie staining):**

100 ml of acetic acid, 500 ml of methanol and 1 g of Coomassie Blue R250 were dissolved in autoclaved ddH_2O to a final volume of 1000 ml.

• **SDS gel destaining solution:**

200 ml of methanol and 100 ml of acetic acid were dissolved in autoclaved ddH_2O to give a final volume of 1000 ml.

• **SYS (BBL) medium:**

10 g of soyhydrolyzate, 5 g of yeast extract and 5 g of NaCl were dissolved in 800 ml of autoclaved ddH_2O. After the pH was adjusted to 7.0 with 1M KOH or NaOH, the final volume was supplemented to 1000 mL with autoclaved ddH_2O. Autoclaved at 121 ° C for 30 min.

• **SOC medium:**

After dissolving in autoclaved ddH$_2$O of 20 gr bactotripton, 5 gr bacto yeast extract, 2 ml of 5M NaCl, 2.5 ml of 1M KCl, 10 ml of 1M MgCl$_2$, 10 ml of 1M MgSO$_4$ 800 ml, Autoclaved for 30 min. at 121℃; After removing the solution from the autoclave, it was expected that the temperature was reduced to 50 ° C. Then, 20 ml of 1M glucose was added under sterile cabinet and left at + 4 ° C.

- **MMA medium:**

1.952 gr MES and 10 ml 1M MgCl$_2$ were dissolved in 800 ml of autoclaved ddH$_2$O. After adjusting to pH 5.8 with 1M KOH or NAOH, the final volume was then supplemented to 1000 mL with autoclaved ddH$_2$O. Autoclaved at 121 ° C for 30 min.

- **100 mM stock solution of acetosyringene (AS):**

0.3924 gr Acetosyringene was weighed and dissolved in 12 ml of 95% ethanol and 8 ml of bi-distilled water was added to the final volume of 20 ml. For 1L MMA 150μl Acetosyringene is required.

- **1X PBS tablet solution:**

1 tablet was dissolved in 100 ml of autoclaved ddH$_2$O in PBS (containing 137 mM NaCl, 2 mM KCl and 10 mM phosphate buffer).

- **Column Equilibration Buffer:**

(1x PBS/0,5M NaCl) 5 of PBS tablet dissolved in ddH$_2$O to obtain 500mL 1xPBS solution. 14,61 G. NaCl added to 500mL 1x PBS and dissolved.

- **High Salt Wash Buffer:**

(50mM TRİS/2M NaCl pH:8.0) 0,6057 G. Tris and 11,688 G. NaCl weighted and dissolved in 80mL ddH$_2$O and adjust pH to 8.0. Final volume adjusted to 100mL with ddH$_2$O.

- **Elution Buffer:**

(200mM Glycine/150mM NaCl pH:2,2) 1,50132 G. Tris and 0,8766 G. NaCl weighted and dissolved in 80mL ddH$_2$O and adjust pH to 2,2. Final volume adjusted to 100mL with ddH$_2$O.

- **Neutralization Buffer:**

(2M Tris pH:8.0) 12,114 G. Tris dissolved in 40mL ddH$_2$O and pH adjusted to 8.0 and final volume adjusted to 50mL with ddH$_2$O.

- **Concentration Buffer:**

(20mM Tris pH:7.5/150mM NaCl) 0,242 G. Tris and 0,876 G. NaCl weighted and dissolved in 80mL ddH$_2$O and adjust pH to 7.5. Final volume adjusted to 100mL with ddH$_2$O.

- **RPMI medium:**

5% FBS, 1% PenStrep, 1% NEAA, 1 & GlutaMax and β-ME

3.8. Preparation of Competent Cell

E. coli XL1Blue competent cell preparation*:*

1- 100 μL XL1Blue cells were inoculated in 10 mL LB and incubated for 1 night on a 37 ° C heated shaker.
2- The following day OD was measured. OD = 4.12
3- 412 μL of the culture 1 day old was inoculated into 100 mL LB and left to shake for 3 hours on a 37 ° C heated shaker.
4- Samples were left on ice for 10 min.
5- 1 M CaCl2 (1.47 g CaCl2 + 10 mL ddH2O = 1M CaCl2) was prepared.
6- 10 mL of 0.1 M CaCl2 (1 M CaCl2 + 9 mL ddH2O = 0.1 M CaCl2) was prepared and left on ice.
7- Cells were transferred into a 50 mL beaker and centrifuged at 600 rpm, + 4 ° C for 3 min. Supernatant was thrown.
8- The pellet was resolved by adding 10 mL of cold 0.1 M CaCl2 onto the pellet.
9- After thawing, the mixture was allowed to stand on ice for 20 min.
10- 0.1M CaCl2 / 15% glycerol has prepared.
11- 5 mL of cold 0.1 M CaCl2 / 15% glycerol was added to the mixture on ice and mixed by invert.
12- Aliquoted to 1.5 mL Eppendorf tubes in 100 μL and left at -80 ° C for freezing.

Preparation of Agl1 competent cells containing p-soup:

1- 100 μL Agl1 cells containing p-soup were inoculated with 5 mL LB containing 15 μL of carbamycin and 3 μL of rifamycin in the heated shaker at 28 ° C for 1 night.
2- The following day, OD was measured. OD = 4.152
3- Cells were transferred into a 50 mL falcon tube and left on ice for 5 min.
4- Centrifuged for 5 min. at 3000xg + 4 ° C. Supernatant was thrown. 10% glycerol was added to the pellet and mixed.
5- Centrifuged for 5 min. at 3000xg + 4 ° C. Supernatant was thrown. 2.5 ml of 10% glycerol was added to the pellet and mixed.
6- Aliquoted to 1.5 mL Eppendorf tubes in 100 μL and left at -80 ° C for freezing.

4. RESULTS AND DISCUSSION

4.1. Cloning The Genes

The Pfs48/45 gene was amplified by PCR and verified by electrophoresis as described in material and methods. Verification by electrophoresis results shown in figure 4.1.
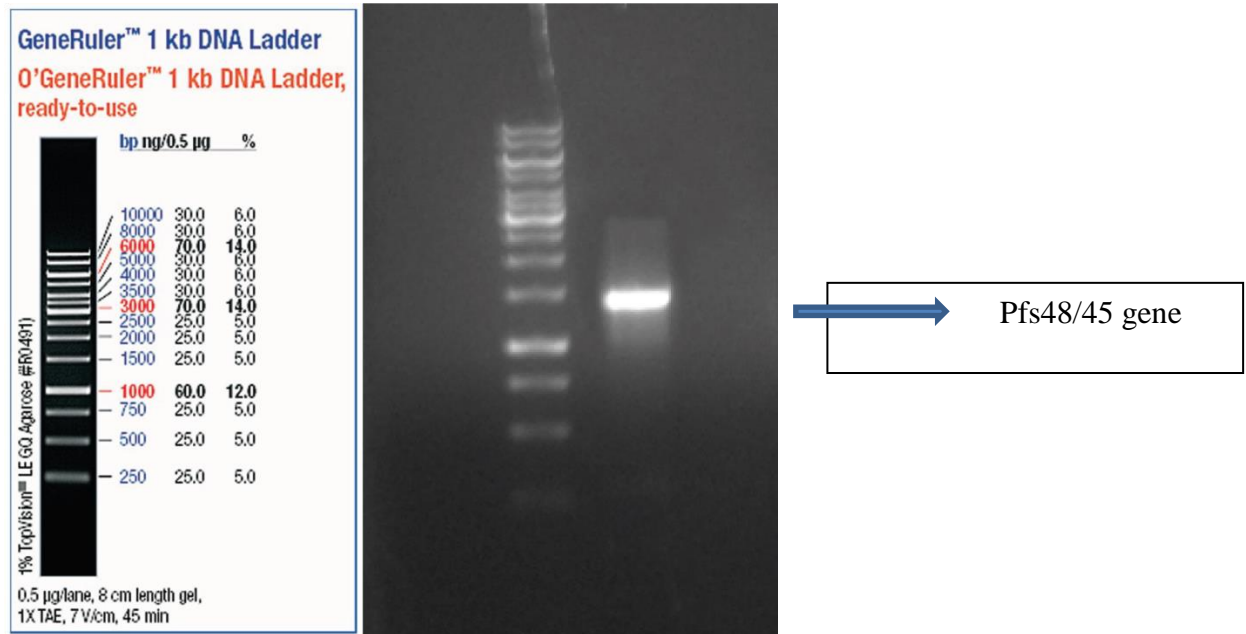

Figure 4.1. Verification of PCR produced gene of Pfs48/45. The band shows correct size of Pfs48/45, successfully amplified by PCR.

The PCR product digested with Age1 and XhO1 enzymes and verified by electrophoresis as described in material and methods. Verification by electrophoresis results shown in figure 4.2
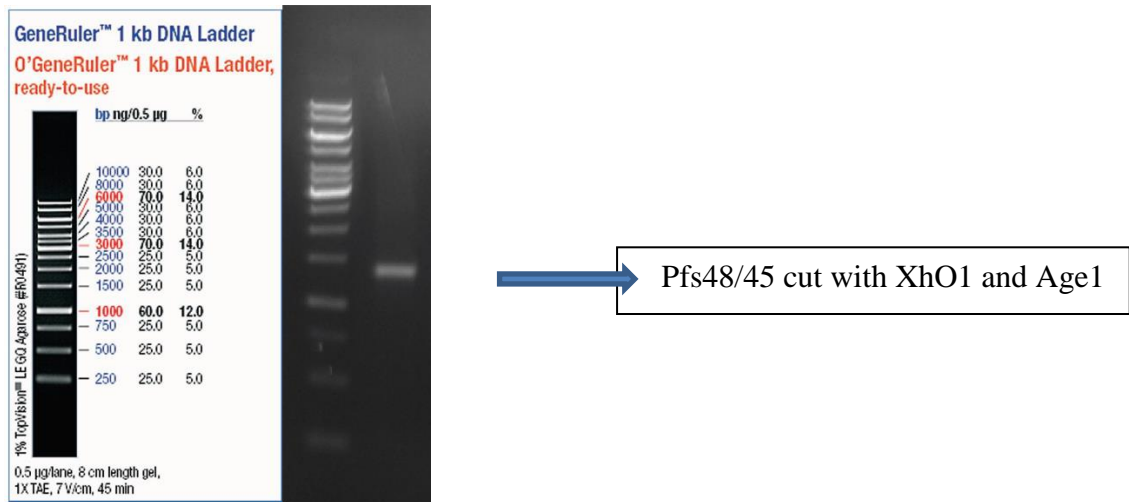
Figure 4.2. Verification of Pfs48/45 digested with XhO1 and Age1 enzymes.

The pEAQ binary expression vector was cut from gel and recovered as described in material and methods. Verification by electrophoresis results shown in figure 4.3
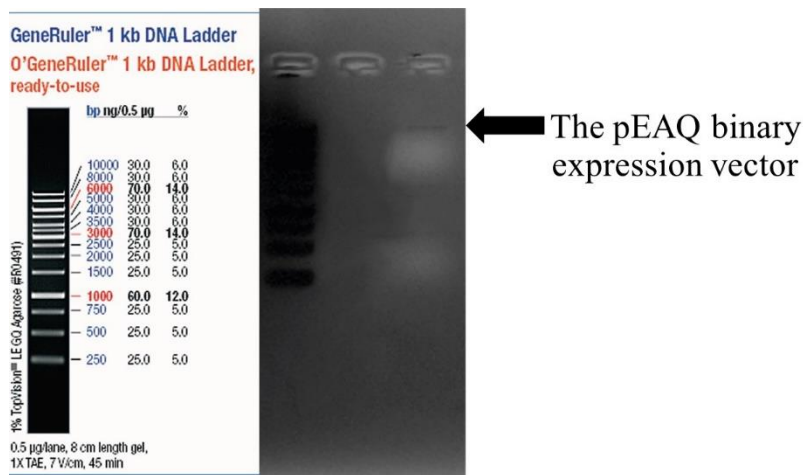

Figure 4.3. Verification of recovery from gel of pEAQ binary expression vector.

pGreen was digested with XhO1 and Pac1 enzymes and recovered from gel as described in material and methods. Verification by electrophoresis results shown in figure 4.4

Figure 4.4. Verification of pGreen digested with XhO1 and Pac1 and recovered from gel.

The modified Endo H and modified PNGase F genes digested with XhO1 and Pac1 enzymes and recovered from gel as described in material and methods. Verification by electrophoresis results shown in figure 4.5
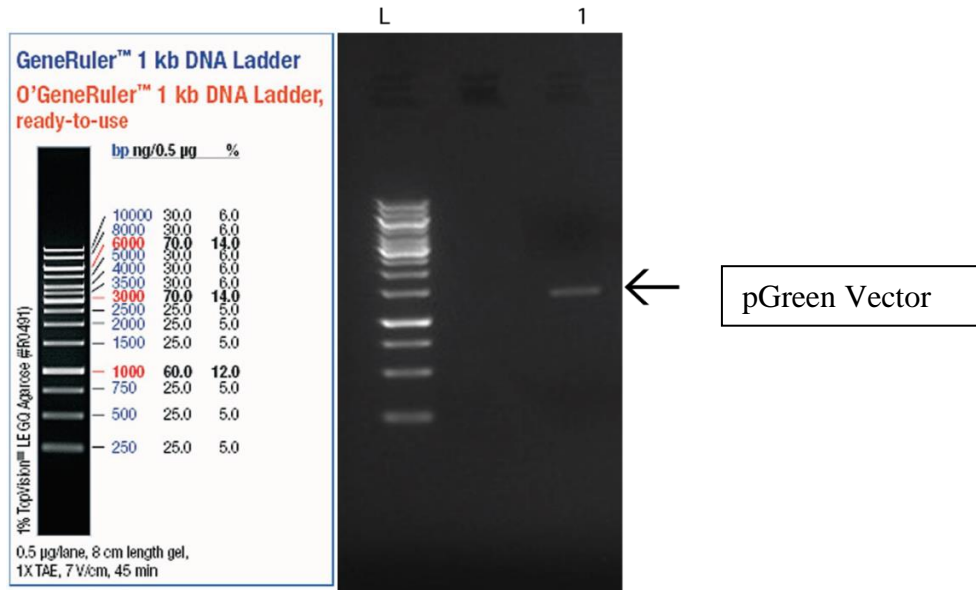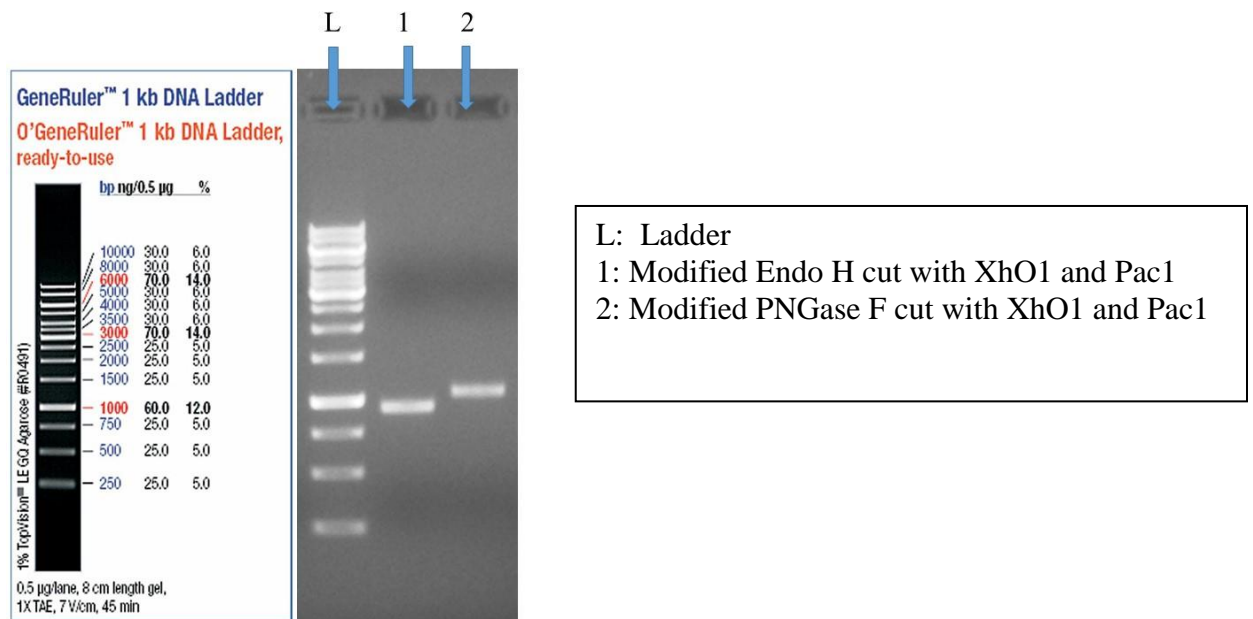

Figure 4.5. Verification of modified Endo H and PNGase F genes digested with XhO1 and Pac1 and recovered from gel.   1: Endo H   2: PNGase F   L: GeneRuler 1Kb Ladder

4.2. *E. coli* **Constructs**

Ligated sample (pEAQ-Pfs48/45) transferred to *E. coli* XL1Blue competent cell by heat-shock method as described in material and methods. After purification, isolated plasmids digested with XhO1 and Age1 enzymes and verified by electrophoresis. Results are shown in figure 4.6
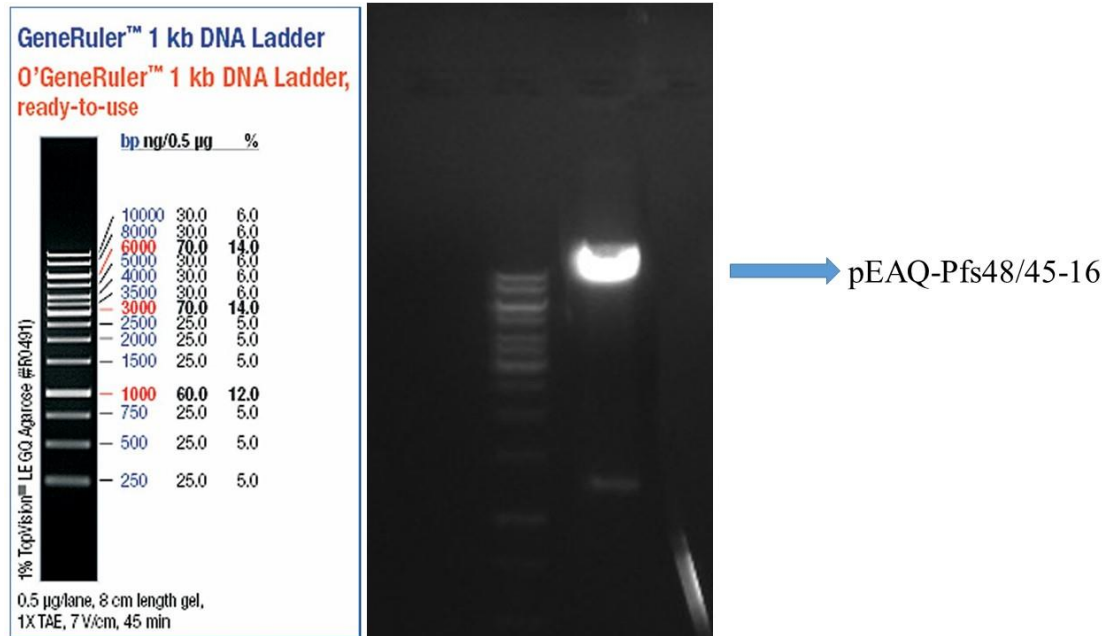

Figure 4.6. Verification of pEAQ-Pfs48/45 gene ligated and digested with XhO1 and Age1 enzymes.

Ligated samples (pGr-Endo H and pGr-PNGase F) transferred to *E. coli* XL1Blue competent cell as described in material and methods. After purification, isolated plasmids digested with XhO1 and Pac1 enzymes and verified by electrophoresis. Results are shown in figure 4.7
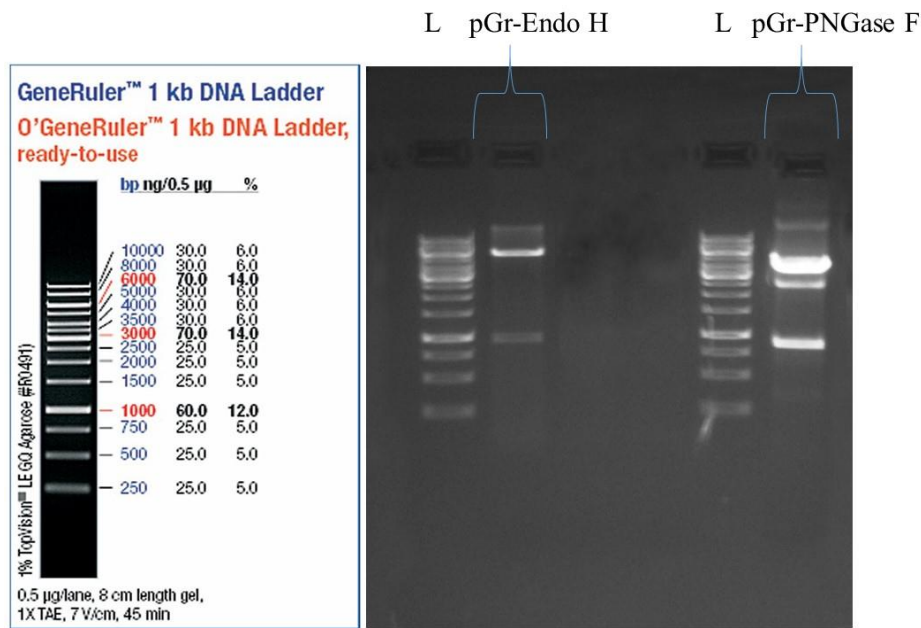
Figure 4.7. Verification of pGr-Endo H and pGr-PNGase F genes ligated and digested with XhO1 and Age1 enzymes.

4.3. Western Blotting

Infiltrated leaves were collected at 7 dpi and analyzed with Western blotting as described in material and methods. As the result of western blotting shown in figure 4.8
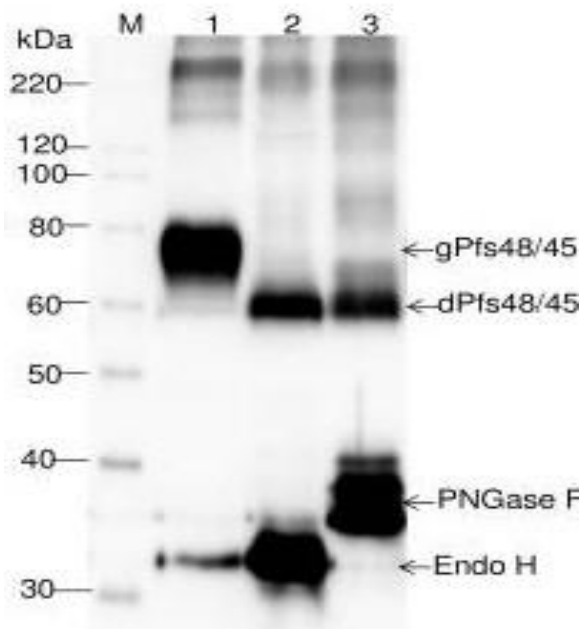

Figure 4.8. Western blot analysis of glycosylated (1: pEAQ-Pfs48/45) and deglycosylated (2: pEAQ-Pfs48/45 / pGr-Endo H and 3: pEAQ-Pfs48/45 / pGr-PNGase F) samples.

4.4. SDS PAGE

Purification of plant produced Pfs48/45 variants (glycosylated and deglycosylated) were performed as described in material methods. SDS Page results for each sample shown in figures 4.9
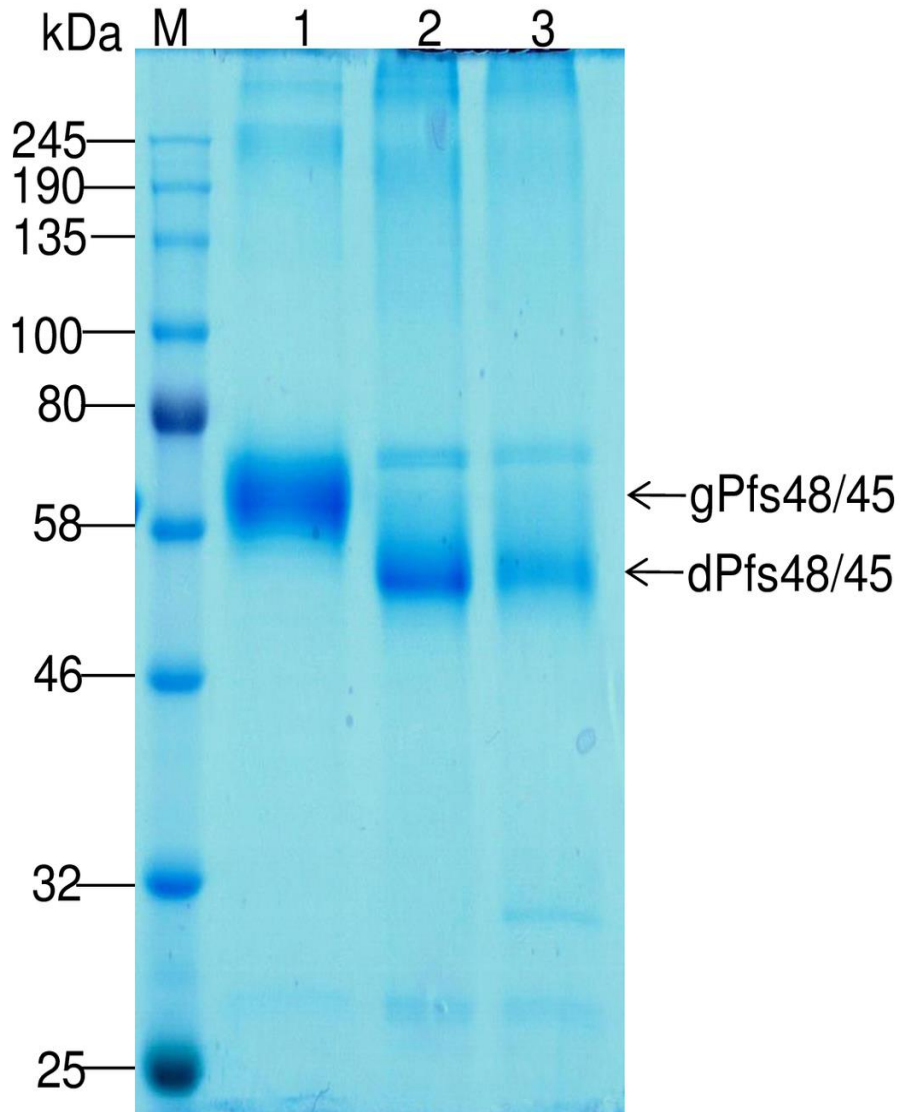

Figure 4.9. SDS-PAGE analysis of purified plant produced Pfs48/45 variants. Lanes were loaded with ~1.0 μg per lane for glycosylated and deglycosylated plant produced Pfs48/45proteins. Lanes: 1- pEAQ-Pfs48/45; 2- pEAQ-Pfs48/45 /pGr-Endo H 3- pEAQ-Pfs48/45 / pGr-PNGase F

4.5. Native PAGE, MRA-26 Antibody/Anti-FLAG Antibody Comparison via WB

Native PAGE is performed using Reduced and Non-reduced samples and running buffers without denaturants or SDS as described in material and methods. Hybridoma medium used as primary antibody and anti-mouse antibody used as secondary antibody for comparing with anti-FLAG antibody. Results shown in figure 4.10
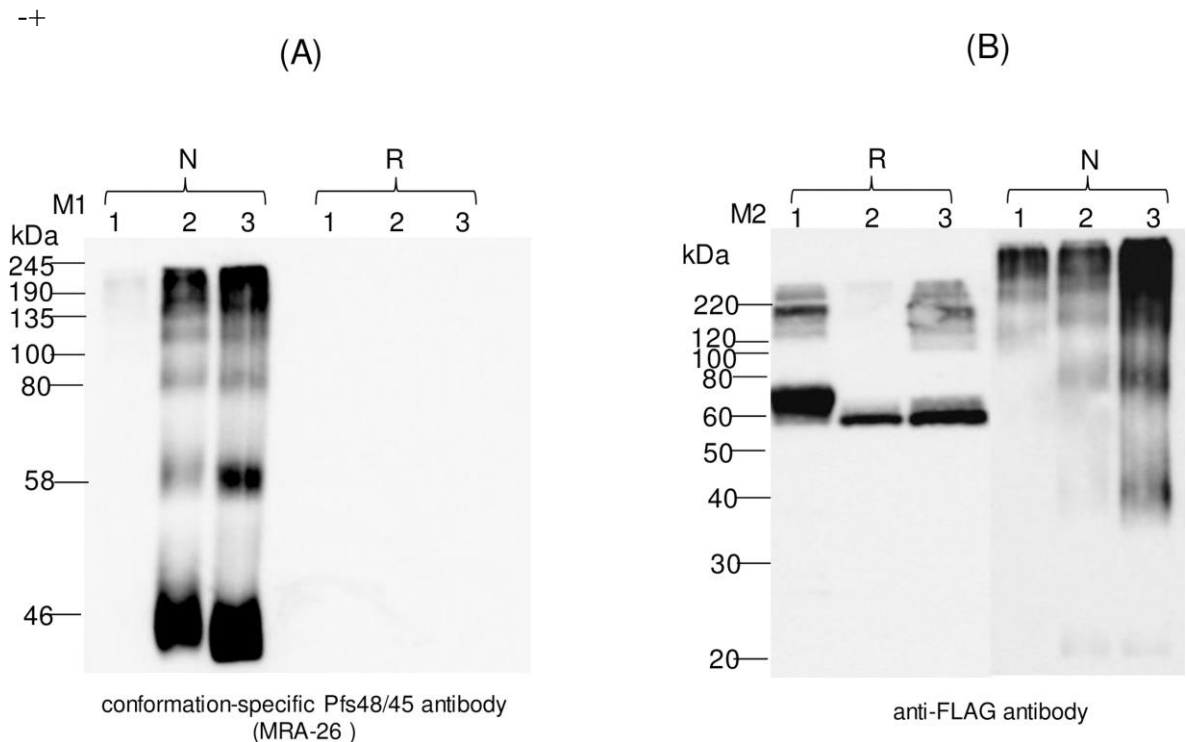

Figure 4.10. Western blot analysis of Pfs48/45 variants using A: Hybridoma Medium (MRA-26 Antibody) B: anti-FLAG Antibody after Native PAGE.

A: Native PAGE followed by western blot analysis of (R)educed and (N)on-reduced samples.   Primary antibody: MRA-26 Antibody   Lanes: 1- pEAQ-Pfs48/45 2- pEAQ-Pfs48/45 / pGr-Endo H   3- : pEAQ-Pfs48/45 / pGr-PNGase F

B: Native PAGE followed by western blot analysis of (R)educed and (N)on-reduced samples.   Primary antibody: anti-FLAG Antibody   Lanes: 1- pEAQ-Pfs48/45 2- pEAQ-Pfs48/45 / pGr-Endo H   3- : pEAQ-Pfs48/45 / pGr-PNGase F

M1: Color Prestained Protein Standard (New England BioLabs)

M2: MagicMark XP Western Protein Standard (ThermoFisher Scientific)

5. CONCLUSIONS

Pfs48/45 is one of the leading TB vaccine candidate against malaria and have an important role in parasite reproduction. Pfs48/45 is a protein which is on the gametocytes, gametes and zygotes of parasite. Pfs48/45 is a complex cysteine rich ((16 cysteines involved in disulfide bond formation) membrane anchored protein and has seven potential N-glycosylation sites. N-linked glycosylation sites can be aberrantly glycosylated in any eukaryotic systems. A challenge to developing a vaccine based on Pfs48/45 is the production of correctly folded recombinant protein. Proper folding of many cysteine-rich proteins, including Pfs48/45, depends on correct formation of disulfide bridges. Currently, several expression systems, bacterial, yeast, mammalian and insect cells cultures have been developed and extensively used for expression various types of pharmaceuticals. Transient gene expression in plants has emerged as an alternative expression system especially for production of proteins that are difficult-to-express proteins and is increasingly being used by industry and academia. This expression platform offers several advantages over other expression systems, including possession of eukaryotic PTM machinery and the ability to accumulate hundreds of milligram quantities of target protein per kilogram of leaf biomass in less than a week. Additional advantages are easy scalability, inexpensive production of valuable recombinant proteins of interest within a short time frame, and an improved safety profile due to lack of endogenous human pathogens. The technology can be particularly useful for expressing glycosylated proteins. However, the ability of plants to glycosylate proteins also can be a significant limitation for expression of some proteins. Recently, a strategy of enzymatic deglycosylation of proteins in plants by co-expressing bacterial PNGase F or Endo H was developed (Mamedov et al., 2012; Mamedov et al., 2012).

In this study, glycosylated and deglycosylated variants of Pfs48/45 protein were produced in *N. benthamiana* plant using above described strategy. We performed cloning, plasmid construction, plant infiltration, protein production, protein isolation and purification of different Pfs48/45 variants as described in Materials and Methods. We show that, in contrast to the glycosylated form, plant produced *in vivo* Endo H deglycosylated Pfs48/45 was recognized by conformational specific Pfs48/45 monoclonal antibody, MRA-26 antibody, in a manner like its PNGase F deglycosylated counterpart. It should be noted that MRA-26 antibody is conformational specific Pfs48/45 mAb antibody, known transmission blocking (TB) antibody. Thus, these results demonstrate that Endo H *in vivo* deglycosylated Pfs48/45 antigen has a potential for the development of a Pfs48/45-based TB malaria vaccine.

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ÖZGEÇMİŞ

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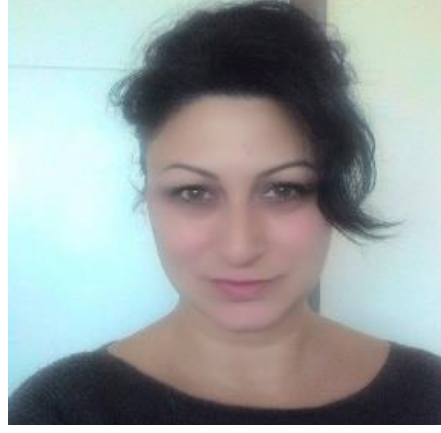
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MESLEKİ VE İDARİ GÖREVLER

ESERLER

Uluslararası hakemli dergilerde yayımlanan makaleler

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