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DEVELOPMENT OF SEROLOGICAL DETECTION METHODS FOR 'CANDIDATUS PHYTOPLASMA PHOENICIUM'

by
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AN ABSTRACT OF THE THESIS OF

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Title: Development of Serological Detection Methods for '*Candidatus* Phytoplasma phoenicium'

'*Candidatus* Phytoplasma phoenicium' has been suggested as the causal agent of Almond Witches' Broom disease (AlmWB), a disease that has devastated hundreds of thousands of stone fruit trees in Lebanon for the past three decades. Thus far, the disease has been identified only in Iran and Lebanon. AlmWB is invasive, suspected of being transmitted by many leafhopper and psyllid species as well as propagative tissue or grafting. This raises the need for strict quarantine measures as the damage of the disease severely impacts yield and kills trees. To date, specific detection of '*Ca*. P. phoenicium' is done by PCR and variants of PCR such as qPCR or nested PCR. Although reliable, these methods are more time consuming, more expensive, and with comparable sensitivity to serological detection methods. Phytoplasmas have not been yet grown in axenic cultures and the expression of their membrane proteins was reported to be very difficult, therefore, no commercial serological tests are available for phytoplasma detection.

This thesis attempted to express 8 previously identified integral membrane proteins in different *E. coli* expression systems and expression vectors; but ultimately no protein expression was observed. Modifications were made to express only the non-transmembrane regions of the targeted proteins with no success. The methodology was shifted towards trying a cell-free expression system which was tested with two transmembrane proteins and one truncated protein excluding the transmembrane region. While all potential possibilities of this system were tried, no expression of the target proteins was obtained.

Finally, we tested three anti-peptide polyclonal antibodies, designed against antigenic and exposed sites of the membrane proteins. Different serological assays were conducted such as Tissue Blot Immunoassay (TBIA), Dot Blot Immunoassay (DBIA), and Western Blot. Only one of the antibodies tested positive in TBIA but similar results were obtained in negative control samples confirmed by PCR tests. This may suggest that the antibodies reacted with either a plant protein, or with an endosymbiont. A specific protein was not observed in western blot, neither was there a positive reaction in DBIA, possibly due to the low titer of phytoplasma in plants. To improve the sensitivity of serological tests, further tests with signal amplifying strategies or with protein concentrating, respectively, are suggested. Plant expression systems may also be recommended.

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ABBREVIATIONS

%	Percent of a Hundred
°C	Degree Celsius
aa	Amino acid
AAP	Acquisition access period
Ab	Antibody
AlmWB	Almond witches'-broom
Amp	Antigenic membrane protein
AMP	Ampicillin
AP	Apple proliferation
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AY-WB	Aster yellows phytoplasma strain witches' broom
Bax	Bcl-2-assoscianted X
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BI-1	Bax inhibitor-1
Blast	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
BTH	Benzothiadiazole
$CaCl_2$	Calcium chloride
cfu	Colony forming unit
CPMU	Circular potential mobile units
CTAB	Cetyl trimethyl ammonium bromide
DAPI	4,6-diamidino-2-phenylindole-2HCl
DBIA	Dot blot Immunoassay
ddiH_2O	Distilled deionized water

DFD	Direct fluorescence detection
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DPO	Dual priming oligonucleotide
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPPO	European and Mediterranean Plant Protection Organization
et al.	et alia (and others)
E. coli	*Escherichia coli*
FDP	Flavescence dorée phytoplasma
GuHCl	Guanidine Hydrochloride
His	Histidine
IAP	Inoculation access period
IdpA	Immunodominant membrane protein A
IgG	Immunoglobulin G
IMP/Imp	Immunodominant membrane protein
inmp	Integral membrane protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISR	Induced systemic resistance
KAN	Kanamycin
kbp	Kilobase pair
kDa	Kilodaltons
KLH	Keyhole limpet hemocyanin
LAMP	Loop-mediated isothermal amplification
LB	Lysogeny broth
LP	Latent Period
LPMU	Linear potential mobile units
MAP	Multiple antigenic peptide
MCS	Multiple cloning site

MLO	Mycoplasma-like organism
mRNA	Messenger RNA
m	Meter(s)
µg	Microgram(s)
µl	Microliter(s)
µm	Micrometer(s)
µM	Micromolar
mg	Milligram(s)
ml	Milliliter(s)
mm	Millimeter(s)
mM	Millimolar
min	Minute(s)
M	Molar
ng	Nanogram(s)
nm	Nanometer(s)
pg	Pictograms(s)
NBT	Nitroblue tetrazolium
Ni-NTA	Nickel Nitrilotriacetic acid
NTP	Nucleoside triphosphate
ORF	Open reading frame
OY-M	Onion yellows phytoplasma strain M
PAa	Phytoplasma australiense' Australian isolate
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pH	Hydrogen potential
PFGE	Pulsed field gel electrophoresis
PMU	Potential mobile units
PTS	Phosphotransferase system
qPCR	Real-time PCR/quantitative PCR

®	Registered trade mark
RBS	Ribosome binding site
rcf	Relative centrifugal force
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolution per minute
rRNA	Ribosomal RNA
s	Second(s)
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
SecY	Protein translocase subunit SecY
SLY	Strawberry Lethal Yellows
sp.	Species
spp.	Several species
SVM	Sequence-variable mosaics
T7RNAP	T7 RNA Polymerase
Taq	Thermus aquaticus
TAE	Tris-acetate-EDTA
TBIA	Tissue blot immunoassay
tblast	Translated blast
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TCA cycle	The citric acid cycle
TEM	Transmission electron microscopy
tuf	Gene encoding elongation factor
U	Unit(s)

UV	Ultraviolet
V	Volt(s)
v/v	Volume to volume
w/v	Weight to volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I
INTRODUCTION

Witches' Broom is a term coined to describe a crowded bundling of shoots on a tree. These symptoms, in addition to other symptoms characteristic of the disease Almond Witches' Broom (AlmWB), were observed in Lebanon and Iran starting in 1990s affecting primarily almond but also peach and nectarine trees. AlmWB disease has killed over 150,000 stone fruit trees since its introduction in Lebanon (Abou-Jawdah et al., 2014), and those symptomatic and not killed offer low and often unmarketable yield with total loss of production in 1-2 years (Molino Lova et al., 2011).

Phytoplasmas are bacterial plant pathogens of the class Mollicutes with genome sizes between 530-1350 kilo base pairs (kbp) (Marcone, 2014). They are pleomorphic and lack a cell wall. Phytoplasma are phloem-limited and more specifically live in the sieve-tubes of the phloem. Many hemipterous insects like planthoppers, leafhoppers and psyllids have been identified as vectors transmitting phytoplasmas in the persistent manner (Weintraub and Beanland, 2006).

These witches' broom symptoms have been previously reported to be caused by phytoplasmas in other woody plants. Hence, upon suspicion of phytoplasma infection, a phytoplasma belonging to the 16SrIX (pigeon pea witches'-broom group) was identified in symptomatic trees and termed '*Candidatus* Phytoplasma phoenicium' ('*Ca.* P. phoenicium'). Further transmission and molecular studies proved that '*Ca* P. phoenicium' was the causal agent of AlmWB disease and classified in the taxonomic subgroup 16SrIX-B (EPPO, 2017)

With no curative treatments available and many vectors suspected of transmitting the disease in a persistent manner, AlmWB will have devastating effects if introduced into areas where it is not already present. For this reason, the European and Mediterranean Plant Protection Organization (EPPO) has added '*Ca.* P. phoenicium' to its A1 list as a quarantine disease, not present in the EPPO region, with high phytosanitary risk and a high likelihood of entry (EPPO, 2017). To quarantine a pathogen successfully, early detection is paramount. Early detection is also key for rouging infected plants to limit disease spread.

Although polymerase chain reaction (PCR) techniques are available for detection of '*Ca.* P. phoenicium', serological methods hold the potential to reduce cost, reduce time and labor, minimize error, be applicable to perform in the field and potentially prove more specific and more sensitive.

Serological methods have been developed for detection of several pathogens, including viruses, fungi and bacteria. However, the inability to grow phytoplasma in axenic culture and the difficulty in expressing their membrane proteins, were limiting factors for production of commercial serological kits for phytoplasma detection. Even though some reports exist on production of antibodies (Abs) against phytoplasma membrane proteins and their use in host–pathogen interaction studies, these Abs have not been suitable for detection tests.

From this premise, this thesis attempts to develop specific and sensitive serological detection methods for '*Candidatus* Phytoplasma phoenicium'.

CHAPTER II
LITERATURE REVIEW

A. Phytoplasma Discovery

Phytoplasmas are a group of obligate plant pathogenic bacteria that belong to the phylum Tenericutes and the class Mollicutes. Phytoplasma lack a rigid cell wall and are non-helical thus rendering them pleiomorphic (Marcone, 2014).

Prior to their discovery in 1967, several plant diseases caused by phytoplasma were erroneously attributed to different pathogens such as viruses (Marcone, 2014). Upon the discovery of these pleiomorphic bodies inhabiting the phloem elements of diseased leaves, they were termed mycoplasma-like organisms (MLOs) due to their resemblance to mycoplasmas (Doi et al., 1967). What confirmed the theory of these MLOs being causal agents of disease were the studies conducted by Ishie et al. (1967) who reported that mulberry dwarf diseased plants recovered upon treatment with tetracycline antibiotics.

In 1994, the terms MLO was replaced with the term phytoplasma (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mollicutes, 1993, 1997). Since their discovery, phytoplasmas have been associated with hundreds of diseases affecting a wide host of economically important plant species (Bertaccini et al., 2014).

The first epidemic of '*Ca.* P. phoenicium' was reported in South Lebanon in the early 1990s (Abou-Jawdah et al., 2002) and in Iran in 1995 (Salehi & Izadpanah, 1995). The spread of the disease was swift in Lebanon spreading from coastal areas to high

elevations, devastating stone fruit production and killing approximately 150,000 trees in a period of 15 years (CABI, 2018).

B. Phytoplasma Taxonomy and Phylogeny

Phytoplasmas belong to Kingdom Bacteria, in the class Mollicutes under the Phylum Tenericutes(Brown, 2010; Brown et al. 2010; Marcone, 2014). They differ from other bacteria by a lack of cell wall and small size.

Mollicutes are thought to have evolved from Firmicutes (Gram-positive bacteria with low G+C content) by degenerative evolution. The closest walled relatives of Mollicutes are *Bacilli* and *Clostridia* species and within Mollicutes, phytoplasmas are most closely related to acholeplasmas (Marcone, 2014). Given the lack of availability to culture phytoplasma axenically, classification has mostly depended on 16S rRNA sequence analysis. Based on these molecular sequences, phytoplasmas have been assigned to 48 species within the provisional genus '*Candidatus* Phytoplasma' (Zhao & Davis, 2016).

The term '*Candidatus*' being introduced by Murray and Stackebrandt (1995) to describe partially characterized, uncultured prokaryotes with available specific molecular data, primarily 16S rDNA sequences.

Species differentiation is based on sequence dissimilarity of 16S rDNA using the standard threshold of 2.5%. Species without these criteria were classified through specific biological and ecological characteristics and other molecular markers (Marcone, 2014).

'*Candidatus* Phytoplasma' belongs to the family *Acholeplasmataceae* under the order *Acholeplasmatales* as proposed in the classification scheme of Martini et al. (2014). Phytoplasmas have been further divided into 33 groups and around 150 subgroups based on 16S rDNA sequences and in-silico RFLP (Bertaccini & Lee, 2018; Zhao & Davis, 2016). The conserved 16S gene, however, has proven over the years to be insufficient to differentiate between closely related strains or variants of a strain. In such cases, less-conserved sequences are analyzed (Marcone, 2014).

'*Ca*. P. phoenicium' has been subsequently placed in 16SrIX group (pigeon pea witches'-broom group) and subgroup 16SrIX-B. Genetic variants of '*Ca.* P. phoenicium' which have been previously described as distinct subgroups 16SrIX-D, -F, and -G (Molino Lova et al., 2011; Wei et al., 2007) are now all unified under 16SrIX-B (EPPO, 2017).

C. Phytoplasma Genome

As other Mollicutes, phytoplasmas have lost a large part of their genome because of degenerative evolution. Genome size of over a 100 phytoplasmas vary between 530 kbps to 1350 kbps, with ranges just as wide in some phytoplasma groups and ranges much narrower in others (Marcone, 2014). Some mycoplasmas with smaller genomes than phytoplasmas have been successfully cultured (Marcone, 2014). This rules out the notion that the small genome size alone might somehow be responsible for the inability to culture phytoplasmas.

Pulsed-field gel electrophoresis (PFGE) has been a very valuable tool to study unculturable phytoplasma genomes. Physical maps of several phytoplasma

chromosomes have been generated by single and double digestions with rare-cutting restriction endonucleases having GC-rich recognition sites and resolving the fragments by PFGE (Marcone, 2014).

To date, only five phytoplasma genomes have been completely sequenced in addition to other draft genomes such as draft genome for '*Ca.* P. phoenicium' and others (Chen et al., 2014; Chung et al., 2013; Mitrovic´ et al., 2014; Quaglino et al., 2015). These sequences include OY-M and AY-WB strains of '*Ca.* P. asteris', PAa and SLY strains of '*Ca.* P. australiense' and AT strain of '*Ca.* P. mali'. Four of the five sequenced phytoplasma genomes have a circular chromosome like most culturable mycoplasmas, whereas '*Ca.* P. mali' has a linear chromosome. The four circular chromosome phytoplasmas show 27-28% GC content whereas '*Ca.* P. mali' displays only 21.4%, which is the lowest of all mollicutes recorded. Finally, four of the phytoplasmas possess one or more extrachromosomal DNA (plasmids) while '*Ca.* P. mali' does not (Macrone, 2014).

Nishigawa et al., (2002) and Ishii et al., (2009) have demonstrated that plasmids of non-insect transmissible lines of phytoplasma lack open reading frames (ORFs) that are present in the insect transmissible lines. This suggests that plasmids have roles in insect transmission. The extrachromosomal DNAs have also been proposed to facilitate integration of genetic material into chromosome (Macrone, 2014).

Each fully sequenced genome has a different number of protein coding regions with assigned protein functions in addition to hypothetical genes. '*Ca.* P. mali' stands out once more from phytoplasma and other bacteria by long terminal inverted repeats

with covalently closed hairpin ends stabilizing the chromosome (Kube et al., 2008, 2012).

Clusters of repeated sequences of up to 20kb lengths in the phytoplasma genome are termed potential mobile units (PMUs) (Macrone, 2014). These PMU clusters are mostly multicopy repeated genes and share features of replicative composite transposons and are postulated to code for candidate virulence proteins (Bai et al., 2006; Hogenhout et al., 2008).

One PMU studied by Toruño et al., (2010) in Aster Yellows phytoplasma strain Witches' Broom (AY-WB), termed PMU-1 with a 20kb size an coding for 21 genes (including replication and membrane targeted proteins), was found to exist both in linear form (LPMU) in the chromosome and in circular form (CPMU) extrachromosomally. The study further showed that CPMUs copy numbers are five-fold more in insect than in plant, and gene expression was also higher in insects. It also reports that a large part of AY-WB's virulence genes are located on PMU loci. This study demonstrated PMU presence in AY-WB chromosome and its significance for phytoplasma virulence. PMUs appear to be unique to phytoplasmas and the number of PMUs and degenerate PMU-like regions varies between different phytoplasma species (Marcone, 2014).

Other studies have shown genetic elements similar to PMUs, termed sequence-variable mosaics (SVMs) form large parts of phytoplasma genomes and are remnants of *Caudovirales* phage attacks in several phytoplasmas which might have even played a role in the evolution of the phytoplasma (Jomantiene & Davis, 2006; Wei et al., 2008b). Plasmids, PMUs, and SVMs can help explain the variability in phytoplasma genomes and their vast presence in diverse environments and hosts (Marcone, 2014).

Another characteristic of phytoplasma genomes, is the loss of many biosynthesis genes such as those necessary for synthesis of many vital compounds such as amino acids, nucleotides, and many biochemical pathways such as TCA cycle. But most importantly, is the absence of phosphotransferase system (PTS) and F-type ATPases gene and subsequently, the ability to synthesis ATP as previously thought (Marcone, 2014). However, Siewert et al. (2014) have identified through an analysis of gene expression of '*Ca.* P. mali' an alternative ATP synthesis pathway. In this pathway malate is taken up and acted upon by multiple enzymes to eventually yield ATP. The crucial enzymes in this process are malate dehydrogenase and acetate kinase. Malate dehydrogenase has been identified in all phytoplasma full and draft genome sequences but not in any other culturable mollicutes. Despite the absence of PTS, glycolysis remains the main energy-yielding process for most phytoplasmas with the known exception of '*Ca.* P. mali', which has a rudimentary glycolysis completely lacking the energy-yielding component (Kube et al., 2008, 2012, 2014; Siewert et al., 2014).

Gene expression of phytoplasma is strongly host-specific and changes drastically upon switching between plant and insect hosts. These changes are usually survival oriented such as five-fold increased expression of the gene coding for large-conductance mechanosensitive channel (which is necessary to survive osmotic pressure in sap) in plants and increased expression of zinc uptake proteins in insects where zinc is less readily available (MacLean et al., 2011; Oshima et al., 2011)

As a final remark of phytoplasma genomes, they code for a multitude of efficient transporter systems that are necessary for the uptake of essential nutrients and many are especially well suited for nutrients present in abundance in plant sap and insect haemolymph (Marcone, 2014).

In 2015, a draft genome of '*Ca*. P. phoenicium' strain SA213 was generated by Illumina sequencing. 78 contigs (354kbp, 26% GC) were assigned to the taxon '*Ca.* Phytoplasma' using the MEGAN approach. These contigs included 333 protein coding sequences. The draft genome is reported to be "far from complete". It was predicted by tblastn that 34 proteins are unique for '*Ca.* P. phoenicium' and 19 of the proteins were predicted to be membrane proteins. A putative inhibitor of apoptosis-promoting Bax factor, termed BI-1, was one of the integral membrane proteins identified and suggested to have a role in enhancing phytoplasma fitness by interfering with host defense mechanisms. Phylogenetic analysis and tblastn results suggest a close relationship between '*Ca.* P. phoenicium' and '*Ca.* P. pruni' (Quaglino et al., 2015).

D. Phytoplasma Morphology

Phytoplasmas have been observed under the transmission electron microscope as generally spherical or ovoid figures sometimes forming chain-like structures, as well as pleiomorphic bodies in between the narrow opening of the sieve plates. Their pleiomorphic nature is due to the absence of a rigid cell wall, with only a single cell membrane (Hogenhout et al., 2008). They have been observed in budding form and presumably undergoing binary fission and different sizes ranging from 80 to 900 nm have been documented with an average of 400 nm (Marcone, 2014). The population of phytoplasma in sieve tube elements can be high or low as a function of colonization patterns and the host species (McCoy et al., 1989).

Marcone (1996, 2014) also studied phytoplasma with the scanning electron microscope, finding that phytoplasma took on a plethora of pleomorphic shapes

resembling “spherical, budding, dimpled and dumbbell shaped cell, and filamentous branching forms present only in tube sieve elements of infected plants”. He postulated the differences in morphology are due to the observation of different phytoplasma developmental stages that are reliant on nutrition, age and number of the phytoplasmas observed. It is also suggested that phytoplasmas may pass from crowded cells to less crowded ones based on observation of phytoplasmas crowding around sieve plates with a large number on one side and fewer on the other. In 1996, Marcone also claimed the phytoplasmas are attached to the inner surface of the host cell cytoplasm membrane and this feature could play a role in pathogenicity.

E. Phytoplasma Ecology

Phytoplasmas generally reside in the plant phloem sieve tube elements and spread systemically in the phloem through the sieve pore plates. Occasional infection of phloem parenchyma and companion cells have also been reported, but the mechanism of their movement is unknown as it is doubted they pass through the 3-4 nm sized plasmodesmata. Changes in plasmodesmata-pore ultrastructure of infected plants have not been reported, and no common motif for movements proteins in phytoplasma have been identified to postulate the use a mechanism similar to viruses (Christensen, 2005).

Some phytoplasmas are eradicated in aerial parts such as ‘*Ca.* P. mali’ in apple trees due to degeneration of the phloem tissue in the winter during which phytoplasma overwinter in roots and spread up to the shoots again in spring (Seemuller et al., 2018).

Sieve elements are largely void of cell organelles, offering less resistance to assimilate flow. They also have high turgor pressure and are rich in nutrients and chiefly

carbohydrates with 10-30% sucrose (Evert, 1977). Companion cells offer similar conditions (except for the presence of cell organelles) and are thus considered also a suitable host for phytoplasma, whereas parenchyma cells are not because of their low turgor pressure (Marcone, 2014).

F. Phytoplasma Transmission

Phytoplasmas are transmitted between plants by phloem-feeding insects of the order Hemiptera, chiefly leafhoppers (Cicadellidae) and planthoppers (Fulgoroidea), and less often psyllids (Psyllidae) in addition to some heteropteran species of the family Pentatomidae (Stinkbugs) (Weintraub & Beanland, 2006).

Insects acquire the phytoplasma from the phloem sap of infected plants during an extended period called acquisition access period (AAP). AAP usually takes a few days depending on the vector and phytoplasma and has even been reported to take a few hours for some species. Once acquired, there is a latent period (LP) during which the phytoplasma must cross the mid-gut membrane to enter the nutrient rich haemolymph. The LP is dependent on the phytoplasma, the vector, and temperature. It can last anywhere between 12 days to 3-5 weeks and even longer. Once inside the haemolymph, they multiply and invade numerous internal organs, the most important being the salivary glands through which they pass along with saliva into the sieve tube elements of the new host during feeding, called inoculation access period (IAP), for which a few hours are enough (Bosco & Tedeschi, 2013; Hogenhout et al., 2008; Pagliari et al., 2019).

Vector specificity varies among phytoplasmas. Some may have low specificity and can be transmitted by many vectors such as 16SrI-B phytoplasmas, while others have high specificity and can be transmitted by only one or a few vectors such as the AP group temperate fruit tree phytoplasmas. Some vectors can transmit several phytoplasmas (Marcone, 2014). Subsequently, the number of insect vectors and their feeding behavior are major determining factors of a phytoplasma's host rage. There is also evidence that some phytoplasmas are transovarially transmitted to insect progeny. Arismendi et al. (2015) estimated that only 4% of phytoplasma insect vectors have been reported to exhibit transovarial transmission. A recent study by Mittelberger et al. (2016) reports transmission of '*Ca.* P. mali' by *Cacopsylla picta* to their eggs, nymphs and F1 adults.

Phytoplasma DNA has been detected in various seeds of phytoplasma-diseased plants. There is conflicting literature suggesting their seed transmission, Faghihi et al. (2011) reported that '*Ca.* P. aurantifolia' is not seed transmitted although it might affect seed germination and seedling growth. Dickinson et al. (2013) claimed that seed transmission of viable infectious phytoplasmas have not yet been reported in refereed papers and the entire status is unclear. On the other hand, reports such as Calari et al. (2011) cited by Bertaccini et al. (2018), claim "it is possible to assume that a low percentage of seed transmission should be considered" based on the detection of phytoplasma in seedlings germinated from seeds of phytoplasma infected hosts.

While phytoplasma are not sap transmissible, they can be transmitted by vegetative propagative tissue and grafting (Marcone, 2014). Young almond and nectarine seedlings (1-2 years old) grafted with AlmWB infected scions in July developed symptoms around 3 months post-inoculation (unpublished results).

Periwinkle (*Catharanthus roseus*) plants are often used as experimental hosts in which phytoplasma titer can be maintained by routinely grafting infected material. Several phytoplasmas have been transmitted by dodder to periwinkle from natural infected hosts (Marcone, 2014).

Although '*Ca.* P. phoenicium' has also been detected in *Cixius* sp. and *Eumecurus* spp. in almond and peach orchards and their surroundings, transmission trials revealed it can be transmitted by *Asymmetrasca decedens*, *Tachycixius viperina*, and *Tachycixius cypricus* (Abou-Jawdah et al., 2014., Tedeschi et al., 2015.). *A. decedens* is reported to be responsible for almond to almond transmission while *Tachycixius* spp. for weeds to almond transmission (CABI, 2018).

'*Ca.* P. phoenicium' appeared around the same time in Lebanon and Iran, and it is unlikely that it spread through insect vectors because of the distance between the two countries and the absence of the disease in countries between them such as Syria and Iraq where almond orchards are widely present. It is rather postulated that the spread between countries was due to human introduction of infected seedlings (CABI, 2018).

G. Phytoplasma Distribution and Hosts

The majority of plants affected by phytoplasma are angiosperms. Although phytoplasmas are present around the world, different taxonomic groups and subgroups of phytoplasmas are distributed in different geographic regions, and usually correlated with the distribution of their insect vectors and host plants. An example of this would be the worldwide occurrence of 16SrI-B phytoplasma and in contrast, 16SrI-L and 16SrI-M are limited to Europe (Marcone, 2014).

'*Ca.* P. phoenicium' is widespread in Lebanon and Iran, and not yet reported in any other countries including almond cultivating countries between Lebanon an Iran. In Lebanon, the disease is present in isolated wild trees, and both well-managed and abandoned orchards. It is present in all areas from coastal areas all the way to high mountainous areas with elevations above 1200m (Abou-Jawdah et al., 2002; CABI, 2018).

Another trait that varies amongst phytoplasma is host specificity. Whereas some phytoplasma have more than 80 plant species as hosts (Ex: 16SrI-A, -B, and -C), others appear to infect only one host (AP group fruit tree phytoplasma). Virtually all phytoplasmas can infect and induce symptoms in periwinkle suggesting host specificity is not set in stone. It is clear, however, that the mechanism of host specificity is not yet well explored. The natural process depends on interactions of disease, insect, and host (Marcone, 2014).

A single plant can be infected by several different phytoplasmas. In addition, several different phytoplasmas can induce the same symptoms in a host. It has also been shown that differences at the level of different strains of phytoplasma can have a strong influence on significant traits such as virulence. Infection of trees with multiple strains of phytoplasma has been documented and shown to have an effect on disease development through antagonistic effects that alter virulence by shifting strain populations (Marcone, 2014).

The primary hosts of '*Ca.* P. phoenicium' (16SrIX-B) in Lebanon are *Prunus dulcis* (almond) (Abou-Jawdah et al., 2002) with some varieties more susceptible that others and *Prunus persica* (peach) (Abou-Jawdah, Sobh, & Akkary, 2009). *Prunus*

armeniaca (apricot) was also recently reported as a host in Iran (Salehi et al., 2018) even though it was previously thought to be a non-host by grafting trials which also suggested plum and cherry are not affected by the disease (Abou-Jawdah et al., 2003). As Salehi et al. (2015) suggest, this might me the result of adaptation of the pathogen to new fruit trees. In Iran, it has been identified on other hosts such as *Prunus scoparia* (wild almond) and GF-677 (*P. amygdalus* × *P. persica*) but peculiarly not on peach (Salehi et al., 2015). Thus far identified wild hosts of the phytoplasma include *Anthemis* spp., *and Smilax aspera* (Tedeschi et al., 2015) and they are preferred by cixiid vectors of '*Ca.* P. phoenicium' while the leafhopper vectors prefer feeding on *Prunus* spp. (Abou-Jawdah et al., 2014; CABI, 2018; Salehi et al., 2015).

H. Phytoplasma Symptoms

Phytoplasma infection can result in a number of symptoms in host plants which vary as a result of multiple factors including the strain of phytoplasma, the host, environmental conditions, age of host, titer of phytoplasma, and the phase of the disease. In Angiosperms, which account for the majority of phytoplasma hosts, specific symptoms include phyllody, big bud, virescence, flower proliferation, witches' broom, off-season growth, shorter internodes, etiolation, and phloem browning. The flower abnormalities result in sterility. Other non-specific symptoms include leaf roll, chlorosis, leaf curl, leaf reddening, smaller leaves, stunting, and vein clearing among others. Recovery of infected plants and plants that remain non-symptomatic but infected over their life-span have also been reported by multiple sources (Macrone, 2014).

A few gymnosperm families have also been identified as phytoplasma hosts. The symptoms reported include chlorosis, stunting, shoot proliferation, and dwarfed needles (Macrone, 2014).

In the case of AlmWB, symptoms on almond trees are primarily proliferation of the shoots on the main trunk with witches' broom occurrence, proliferation with numerous axillary buds appearing perpendicularly on branches with small and chlorotic leaves, and general decline and dieback. After 1-2 years from symptom appearance, production ceases entirely (Abou-Jawdah et al., 2002).

As for nectarine and peach, symptoms progress with early flowering, earlier development of buds on infected branch, appearance of phyllody at flowering, witches' broom on the trunk and crown of trees, and serrate, slim, light green leaves. The main difference between AlmWB on almond and peaches/ nectarine is the absence of phyllody in almond (CABI, 2018).

Young almond and nectarine seedlings (1-2 years old) grafted with AlmWB infected scions develop symptoms around 3 months post-inoculation (unpublished results).

I. Phytoplasma-Host & Vector Interactions

Phytoplasma-host interactions is an area of growing knowledge. Of the most apparent effects of phytoplasma infection is the impairment of phloem transport between source and sink. Other apparent physiological disturbances that might explain symptoms include reduced photosynthesis, pigment quantity, stomatal function, transpiration, root respiration, phytohormone balance, and altered secondary

metabolism. Although the exact mechanisms of these interactions are not yet well-understood, the regulation of many host genes upon phytoplasma infection have been reported (Macrone, 2014).

Moreover, certain effector proteins secreted by phytoplasmas have been shown to be crucial in down-regulating certain genes involved in phytohormone balances and floral abnormalities among other host functions (Macrone, 2014). An effector protein SAP11 has been identified to be produced by phytoplasma in phloem cells. SAP11 is transported to other parts of the plant where it targets plant cell nuclei. Transgenic *Arabidopsis thaliana* expressing SAP11 showed symptoms resembling *A. thaliana* infected with Aster Yellows Witches' Broom phytoplasma (Bai et al., 2009; Sugio et al., 2011).

The effect of phytoplasma on insect vectors ranges from devastating (halving life span, damaging health, and reducing fecundity) to beneficial (increased life span and fecundity, allowing them to live and reproduce on non-hosts, and vector attraction in infected hosts) depending on the phytoplasma, the vector, and the host plant (MacLean et al., 2014; Macrone, 2014; Sugio et al., 2011)

The interaction of a surface membrane protein present abundantly on phytoplasma and termed Antigenic membrane protein (Amp) with insect intestinal membrane microfilaments (actin, myosin, ATP synthase) has been reported in phytoplasma vector species but not in non-vector ones (Galetto et al., 2011; Rashidi et al., 2015; Suzuki et al., 2006). Therefore, the interaction between Amp and insect proteins is suggested to be a significant determinant of vector specificity.

J. Phytoplasma Management

Phytoplasmas are notoriously difficult to control, with existing control measures often do not yield satisfactory results. This fact, along with their devastating effects and invasiveness/ease of spread, has led many phytoplasmas to be placed on quarantine lists to avoid their introduction into areas, where they are not present. AlmWB, for example, is placed on the European and Mediterranean Plant Protection Organization's (EPPO) A1 quarantine list. Quarantine and avoidance strategies are strongly recommended for phytoplasma diseases.

1. Resistance

Phytoplasma resistant plants would naturally be an ideal solution but commercial lines with resistance and good horticultural properties are yet to be available. Nonetheless, research for phytoplasma resistance has been active. One of the very few successful trials was reported by Seemüller et al., in 2018 where the resistant apomictic *Malus sieboldii* was grafted onto M9 rootstock. The trees were evaluated for 6 years in the orchard and a single cross termed 4608 x M9 was shown to have satisfactory resistance and pomological qualities similar to M9.

Grafting trials by Abou-Jawdah et al., (2003) indicate that plum, cherry, and apricot are resistant to AlmWB but there are reports from Iran (EPPO, 2017) that AlmWB does, in fact, infect apricot. Tawidian et al., reported in 2017, the potential of grafting infected almond scions on apricot and plum varieties and observing recovery but the results are only preliminary.

2. Recommended Preventive Measures

Some practices that might lessen the probability of infection include practices such as avoiding planting in areas known to be infected with phytoplasma, using certified disease-free planting materials, scouting for insect vectors and spraying insecticides preemptively, eradication of all hosts of the disease including weeds, which have been found to be phytoplasma hosts by several reports.

Although these cautions are recommended, they usually yield a modest reduction in disease incidence and are often unsatisfactory (Seemuller et al., 2018).

3. Induced Plant Resistance

Several recent studies have reported induced systemic resistance (ISR) and systemic acquired resistance (SAR) to provide resistance to phytoplasma infection, and in some cases, even recovery. An example of ISR would be the study by D'amelio et al. (2011) in which *Chrysanthemum carinatum* plants inoculated with a rhizobacterium and an arbuscular mycorrhizal fungus resulted in reduction of symptomatic plants upon inoculation with chrysanthemum yellows phytoplasma from 93% in control to 73% in treatment in addition to a 70% increase in biomass of the treated plants.

An example of SAR is the study by Wu et al. (2012) in which salicylic acid (SA) was applied to tomato plants before grafting them with potato purple top phytoplasma infected scions. This resulted in a significant drop of percentage of infection from 94% in control to 44% in SA treated. Symptoms of infected plants were also less severe in SA treated plants. Other studies also show promising results with SA or its analogue Benzothiadiazole (BTH) (Miliordos et al., 2017).

4. Disease Suppression/Recovery

Tetracycline antibiotics are famously known to suppress phytoplasma in plants. But their effect is only short-termed. So repeated applications of antibiotics are needed to only suppress the disease. Generally, the use of tetracycline in fruit tree production has been discontinued for several reasons (Seemuller et al., 2009; Zamharir, 2011). Some studies have explored other compounds preliminarily, but no concrete results have yet been presented such as the disappearance of phytoplasma after applications of pyrithione in in-vitro studies (Aldaghi et al., 2008)

Interestingly, some instances of spontaneous recovery (remission of symptoms) from phytoplasma infection have been reported. 'Barbera' is a common grapevine cultivar which is extremely susceptible to Flavescence dorée phytoplasma (FDP), but it also has a high rate of recovery. Research teams such as Margaria et al. (2013) have been investigating the proteomics behind this phenomenon.

K. Phytoplasma Detection and Diagnosis

Considering the difficulty of phytoplasma control, fast, inexpensive, and accurate detection is key to control the spread of the disease. Until recently, phytoplasmas have not been successfully cultured axenically. This rules out isolating and culturing phytoplasma from diseased plants as an identification tool. Contaldo et al. reported in 2016 their success in axenically culturing phytoplasma using CB and Pivs® media. However, they have not released full protocols on how to repeat their results. Bertaccini et al. (2018) describe this system as needing further research to be optimized and consider it a "prospect" rather than presently available tool.

1. Microscopy

The earliest method to detect phytoplasma and diagnose phytoplasma diseases, which was the only primary method until the early 1980s, was observation of ultrathin phloem sections under the transmission electron microscope (TEM). The need for TEM equipment and hassle to prepare ultrathin sections, in addition to the very general non-specific nature of detection have made this approach unattractive for diagnosis with the emergence of better techniques in the modern age. It remains in use, however, for studies on morphology and distribution within hosts (D'amelio, 2011; Musetti, 2004).

In 1981, Namba et al. introduced direct fluorescence detection (DFD) of autofluorescence of necrotic phloem cells of phytoplasma infected plants by a reflection fluorescence microscope in contrast to no such fluorescence in healthy plants. In 1986, Hiruki and da Rocha introduced 4,6-diamidino-2-phenylindole-2HCl (DAPI) fluorescent staining method which fluoresces DNA in phloem cells when observed under TEM, the presence of high fluorescence in the usually not high in nucleic acid phloem sieve tube elements would signify the presence of phytoplasma (or another microorganism). A method using multiphoton confocal laser scanning microscopy paired with the specific vital dyes DiOC7 (3,3'-diheptyloxacarbocyanine iodine) or SYTO 13 (green fluorescent nucleic acid stain) was also developed by Christensen et al. (2004) to visualize phytoplasma cells in living plant tissue.

TEM, DFD, and DAPI are sometimes still in use for preliminary confirmation of phytoplasma presence before progressing to more specific detection methods.

2. Molecular Methods

Since the early 1990s, DNA-based molecular methods became the leading technique in phytoplasma detection. Kirkpatrick et al. (1987) introduced a DNA hybridization for phytoplasma detection and in 1991, Deng and Hiruki introduced PCR detection of phytoplasma targeting 16S rRNA gene which has since become the standard in phytoplasma diagnosis (Maejima, 2014). Any technique depending on molecular methods should be based on proper plant tissue sampling and reliable nucleic acid extraction since phytoplasmas are unequally distributed in the host in terms of both localization and titer (IPPC, 2016).

Phytoplasma titer and location are important considerations for PCR tests as they vary with seasonal changes among other factors. For most phytoplasma, symptomatic leaf petioles, midribs, and stem phloem remain the recommended areas to sample for DNA extraction optimally during summer, however detection has also been shown to be possible from bark or roots of dormant trees.

As for DNA extraction, most methods are well suited for a variety of hosts with certain modification such as a phytoplasma enrichment step for certain woody hosts being reported (IPPC, 2016). To reiterate this, Palmano (2001) published a paper comparing four different phytoplasma DNA extraction protocols and found “little variation” in DNA concentration between methods that does not justify more labor-intensive protocols.

a. PCR

For many specific phytoplasma detection, different specific pairs of direct and nested PCR primers have been designed. Several universal phytoplasma primers were

designed, commonly targeting the 16S rRNA gene. The most common of these primers are the P1/P7 and the R16F2n/R16R2 primer pairs, which are used together in a nested PCR protocol. With universal PCR assays, there comes the risk of false positives from closely related bacteria. This can be eliminated by using more specific assays or sequencing the PCR product if the outcome is extremely imperative. Other commonly targeted genes of phytoplasma PCR detection and classification include 23S rRNA, *tuf* , secY, and ribosomal protein genes (IPPC, 2016).

For some hosts and types of tissue, there may be PCR inhibitors in the extracted DNA. For this reason, it is recommended to use an internal control gene of host/vector to verify PCR competency. Inhibitory effects can be overcome by adding Bovine serum albumin (BSA) or further purifying the DNA using Sephacryl® spin columns (IPPC, 2016).

b. Real-time PCR (qPCR)

Several qPCR assays have been designed for phytoplasma commonly targeting either the 16S rRNA or the 23S rRNA genes. qPCR has been reported to be faster, more or as sensitive (depending on phytoplasma and host), more specific, and more enhanced for high throughput analysis (eliminating post-amplification processing) than nested PCR, but requires additional expensive equipment such as a fluorescence detecting thermal cycler. qPCR also allows the quantification of phytoplasma in host tissue which is useful for many plant-pest interaction studies (IPPC, 2016; Ito et al., 2017).

Most phytoplasma qPCR assays are designed with TaqMan® probes with few reports of intercalating dyes such as SYBR® Green (Satta, 2017). Ito and Suzaki (2017) have provided a highly detailed review and evaluation of exiting universal phytoplasma

qPCR primers and probes and have developed their own multiplex qPCR assay using dual priming oligonucleotide (DPO) reverse primers which allow for the detection of phytoplasmas, *Xylella spp.*, and internal plant DNA control within 1 hour. This assay has a minimum detection of 10 pathogen cells, requires only crude extractions, and amplicons are long enough to be sequenced.

c. Loop-mediated isothermal amplification (LAMP)

LAMP is more sensitive and rapid than PCR and requires no DNA purification or expensive equipment (thermal cycler). LAMP assays for phytoplasma detection have been developed since the early 2000s and they are expected to be a rapid and reliable system for field diagnosis. The first commercial LAMP universal phytoplasma detection kit (Nippongene) has been available in Japan since 2011(Maejima, 2014).

Mehle et al., (2017) performed a test performance study of three different LAMP kits developed for grapevine phytoplasma detection. With over 98% accuracy, the tests performed on crude grapevine leaf homogenates were found to have specificity and sensitivity comparable to the most efficient qPCR methods for grapevine phytoplasma detection. LAMP field applicability offer an advantage over PCR methods and can be used in routine fast diagnosis of grapevine phytoplasmas in field or quarantine surveys.

d. '*Ca.* P. pheonicium' detection

As with other phytoplasmas, multiple composite samples (3-4) should be taken from symptomatic branches with phytoplasma being found in petals, petioles, but most concentrated in root and stem phloem. For asymptomatic plants, titer and localization

may be very uneven throughout the plant and thus might result in false negatives (EPPO, 2017).

Detection of '*Ca.* P. phoenicium' relies mainly on PCR and qPCR methods. Conventionally, it relied on the universal P1/P7 and R16F2n/R16R2 primers, but semi-specific PCR primers such as ALW-F2/ALW-R2 that detects 16SrIX group phytoplasma (Abou-Jawdah et al., 2003) and 'Ca. P. phoenicium' specific PCR primers such as AW16sF/AW23sR as well as specific qPCR primers AWsF/AWsR with a TaqMan® probe have been developed (Jawhari et al., 2015).

3. Serological Methods

Serological detection methods have not been as widely relied on for phytoplasma as other plant pathogens such as viruses. This phenomenon is due to the inability to axenically culture phytoplasma cells and difficulties in purifying them. With reports of successful phytoplasma antigen purification and antibody (Ab) production in the 1980s, Enzyme-Linked Immunosorbent Assay (ELISA) kits for phytoplasma detection started being developed such as the aster yellows ELISA test by Lin and Chen (1985) based on monoclonal antibodies raised against partially purified immunogen from leafhopper salivary glands, or "tomato big bud MLO" ELISA kit by Clarke et al., (1989) based on polyclonal and monoclonal antibodies raised against purified phytoplasmal plant extracts as immunogens. In recent literature, the development of phytoplasma specific antibodies have commonly relied on recombinant expression of membrane proteins and more often the immunodominant membrane proteins (IMPs) being abundantly present

on the cell membrane as promising antigens for detection assays (Arashida et al., 2008; Wambua et al., 2017).

a. Immunodominant Membrane Proteins (IMPs)

Kakizawa et al., (2006), classified IMPs based on the location of the coding gene in the genome and the structure of the protein as either (i) immunodominant membrane protein (Imp), (ii) immunodominant membrane protein A (IdpA), and (iii) antigenic membrane protein (Amp). These proteins have been identified in several phytoplasmas over the years and orthologues of these genes have been sequenced and characterized in many other phytoplasmas. Research has shown phytoplasma to possess either one or two IMPs on their surface with Imp coexisting with one of the other IMPs (Siampour et al., 2013).

Konnerth et al. (2016) reported IMPs have a molecular mass between 15.7 and 23 kDa. They also reported, through sequencing of genes surrounding IMPs in different phytoplasmas, that the localization of the genes encoding any of the IMPs is conserved in the phytoplasma genome among different strains. In addition, the predicted transmembrane protein structure was similar for each type of IMP between sub-types but different outside of the sub-type.

Some reports also suggest IMPs can be purified by phase partitioning and centrifugation due to their high abundance (Konnert et al., 2016; Thomas and Balasundaran, 2001).

Amp genes have been reported to be highly variable and subject to strong positive selection, have in-vitro interaction with vector proteins such as myosin, actin, and ATP synthase, as well as being linked with phytoplasma transmission, specificity with in-

vivo studies demonstrating reduced phytoplasma transmission rates upon administration of anti-Amp antibodies to vectors. Imp has been shown to bind to plant actin and has been hypothesized to have a role in phytoplasma motility, while transgenic expression of Imp in plants did not result in any phenotypical change leading to assume it is not an effector protein in pathogenesis (Boonrod et al., 2012; Rashidi et al., 2015; Siampour, 2013).

In the draft genome sequence by Quaglino et al. (2015), 69 out of 336 protein identified were membrane proteins characterized by at least one predicted transmembrane domain. Notable mentions included in the article are the predicted inhibitor of apoptosis-promoting Bax factor termed Bax inhibitor-1 (BI-1) predicted to have a role in phytoplasma ecology, and an integral membrane protein (inmp) which allows differentiation of phytoplasma strains trough unique sequences.

b. Recombinant Membrane Protein Expression

Heterologous expression of membrane proteins is notoriously difficult with expression induction very often being toxic to the expressing cell by overproduction of T7 RNA Polymerase (T7RNAP) triggering ribosome destruction and cell death, and at a second stage by overloading of folding and insertion machinery (Angius et al., 2018; Hattab et al., 2014; Henrich et al., 2015; Miroux and Walker, 1996; Niwa et al., 2015; Wagner et al., 2008).

Hattab et al. (2014) proposed several strategies to overcome this Toxicity in T7 promoter *Escherichia coli* cells ranging from simple manipulation of expression conditions and choice of cells to genetic manipulation of target protein , addition of fusion proteins, and finally to utilizing a cell-free expression system. Apart from

membrane protein toxicity, *E. coli* expression poses other obstacles including inclusion body formation, incomplete synthesis, protease degradation, and others (Hattab et al., 2014).

E. coli remains the most widely successful membrane protein expression system with half of unique membrane protein structures reported being produced in *E. coli*. Other microbial systems commonly used include *Pichia pastoris* and *Saccharomyces cerevisiae.* About 63% of these reported membrane proteins have been expressed under T7 promoter, with Arabinose promoter coming in second at 13-17% and T5 promoter as the third most successful at around 7% (Angius et al, 2018; Hattab et al., 2015; Hattab et al., 2014).

Of the membrane protein structures reported, 28% have been expressed in C41(DE3) and C43(DE3) *E. coli* mutants with integral membrane proteins having great success under the T7 promoter (Hattab et al., 2015) . These mutants were developed by Miroux and Walker (1996) and showed successful overexpression of toxic membrane proteins where it had failed in parental BL21(DE3) strain and where BL21 had succeeded, C41 yielded much higher membrane protein amounts, and C43 yielded even more. These results were confirmed by numerous research publications throughout the years (Anguis et al., 2018; Dilworth et al., 2018; Dumon-Seignovert et al., 2004; Wagner et al., 2008). The commercial OverExpress™ C41(DE3) and C43(DE3) competent cells (Lucigen) claim they are "Effective in expressing toxic & membrane proteins" and "are recommended for membrane proteins with T7 vector" ("OverExpress", 2018).

The C41 mutant has 10-fold less mRNA than its parental strains and C43 mutant has even less and thus might own its tolerance to overexpression complications to a less

extremely active T7RNAP (Anguis et al., 2018). Anguis et al. (2018) recently reported two new mutant strains C44(DE3) and C45(DE3) which they predict "will most likely expand the use of *E. coli* for MP production".

Plasmid choice also influences the outcome of membrane protein expression. Hattab et al. (2015), reported that the most successful membrane protein expression in C41 and C43 cells reported by some of the most frequent published users of these systems were performed with pRSET (Invitrogen) vectors for C41 and pET (Novagen) vectors for C43, although many papers cite successful membrane protein expression with pRSET-C43 combinations (Dilworth et al., 2018; Dumon-Seignovert et al., 2004; Hattab et al., 2014). For T7 promoter based *E.coli* expression, most articles reported using Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducer concentration of 1 mM and a temperature of 37Cº and 30 Cº (Hattab et al., 2018).

Hattab et al. (2014) noted however, that the selection of mutants such as C43 do not always result in successful expression of membrane proteins even at the mRNA level (excluding protein folding or insertion caused toxicity) with noted examples.

If the target membrane protein is expressed but aggregated into insoluble inclusion bodies, these inclusion bodies can be solubilized by a range of ionic and non-ionic detergents such as Sodium Dodecyl Sulfate (SDS), Triton X-100, 8M Urea, 6M guanidine hydrochloride, and others (Dilworth et al., 2018).

Many phytoplasma membrane proteins have been reported to be successfully expressed and used to generate specific polyclonal and monoclonal antibodies. Recent examples include:

(i) Expression of full Imp gene of '*Ca*. P. oryzae' with pQE30 (Qiagen) plasmid in BL21 cells with an insoluble recombinant protein solubilized by 6M guanidinium-

hydrochloride and used to produce specific monoclonal antibodies (Wambua et al., 2017).

(ii) Expression of hydrophilic domain of Amp gene of Japanese Hydrangea Phyllody Phytoplasma expressed in pET (Novagen) system and unspecified *E. coli* strain resulting in a soluble protein to raise specific polyclonal antibodies. The intact Amp gene could not be overexpressed (Arashida et al., 2008).

(iii) Expression of hydrophilic domain of Imp gene of onion yellows phytoplasma using pET30a (+) system (Novagen) in BL21-CodonPlus (DE3)-RIL *E. coli* cells (Stratagene) since the intact Imp gene retarded *E. coli* growth (Kakizawa et al., 2009).

(iv) Expression of intact full-length Imp of lime witches' broom phytoplasma expressed in pQEUA (Qiagen) and M15 *E.* coli (Qiagen) cells and used to raise specific polyclonal antibodies (Siampour et al., 2013).

Galetto et al. (2013) have published a protocol for the recombinant expression of phytoplasma membrane proteins. In line with observations of Arashida et al. (2008) and other publications stated in their protocol, they point out that in some cases the expression of IMPs such as Imp and Amp in their native full-length sequence results in "a significant delay" in the growth rate of the transformed *E. coli*. Konnerth et al. (2016) reported the same observations stating the expression of full-length Imp has resulted in complications such as hindrance of cell growth, protein aggregation or very low soluble protein yields, while the expression of Imp with truncated transmembrane region expressed a highly soluble protein. Only the hydrophilic region of Amp was successfully expressed in *E. coli* in soluble form and expression of full IdpAs yielded much lower proteins than that of the truncated IdpAs. They are all in agreement with the

observation that the deletion of the transmembrane domain of these proteins results in successful expression and the absence of the negative effects. Accordingly, Galetto et al. (2013) suggest the first step to identify and remove the transmembrane coding region of the gene. In their protocol they use the T7 promoter-based pRSET vector system (Invitrogen) transformed in BL21 (DE3) pLysS *E. coli*.

Even though some researchers have reported successful phytoplasma recombinant IMP expression and specific antibody production, the sensitivity of these antibodies is well below the detection limit of molecular techniques. They have proven useful however in other studies such as studying IMP expression patterns in plants, localizing phytoplasma, and protein-protein interactions (Konnerth et al., 2016).

c. Cell-Free Membrane Protein Synthesis

Cell-Free expression systems are in-vitro protein expression systems with all the necessary cell machinery for protein synthesis supplemented with the compounds needed such as NTPs and amino acids. Cell-Free systems are relatively old techniques that have been improved over the years in design, yield, applications and success rate. Cell-Free systems can be developed from lysates of many prokaryotic and eukaryotic cells. Commonly the lysates of *E. coli* have been used in these systems (Henrich et al., 2015),

Problems with using cell lysates include the presence of ribonucleases and proteases in addition to unneeded cell lysate components and debris. To circumvent this issue, the protein synthesis using recombinant elements (PURE®) system was developed that includes 36 recombinant enzymes involved in translation and transcription and highly purified 70S ribosomes. Further developments of this system made all the recombinant

proteins added to the reaction “tag free” facilitating easy purification of synthesized protein (Kuruma & Ueda, 2015).

Cell-Free systems facilitate expression of membrane proteins by eliminating a range of problems associated with in-vivo expression including toxicity, poor solubility, low yield, and ineffective transport and membrane insertion machinery. Cell-Free systems also eliminate the time-consuming practices of cloning genes into expression vectors as they can synthesize proteins directly from special PCR products. Expression of membrane proteins in Cell-Free systems was first reported in the mid-2000s and has been more and more widely employed with varying systems and protocols (Henrich et al., 2015; Niwa et al., 2015).

In the case of membrane proteins, two issues are mentioned: the absence of cell membranes for the membrane proteins to be inserted into and subsequently the irreversible aggregation of hydrophobic membrane proteins. This has been solved by the addition of structures such as lipid vesicles (liposomes) or Nanodiscs (Kuruma & Ueda, 2015; Niwe et al., 2015).

To the best of our knowledge, there have been no reports of phytoplasma protein synthesized in a cell-free system.

d. Anti-Peptide Antibodies

As an effective alternative to heterologous recombinant proteins, short synthetic peptides (10-20 amino acids, standard being 15aa) can be used to raise Abs against a native protein. The resulting anti-peptide Abs have been shown to cross react with the native protein with high frequency and are as effective as anti-protein Abs (Hancock & O’Reilly, 2005; Lee et al., 2016).

Since the short peptides are only long enough to have one antigenic site, the resulting Ab epitope will be well-defined, and it will have the specificity of a monoclonal Ab, while keeping the affinity of a polyclonal antibody, and having a relatively low cost (Voskuil, 2014). The optimal size of the peptide is defined as 10-20aa since shorter peptides might not be long enough to form an epitope and longer ones may assume structural folding conformations different from the native protein (Hancock & O'Reilly, 2005).

The most crucial step in the process is the choice of peptide, because there is no guarantee of the anti-peptide antibody cross reacting with the native protein, with the probability estimated by Hancock and O'Reilly (2005) to be around 50%. There is no proven guideline to designing a successful or high chance of success peptide. Rather, there exist multiple tools such as B-cell epitope prediction computation and antigenicity predictors, and native protein structure predictors (especially in the case of unsuitable transmembrane domain predictors for membrane proteins) that will improve the likelihood of cross reactivity. Another method of increasing the likelihood of success is to immunize with multiple peptides from the protein sequence or try the peptide in different animals (Hancock & O'Reilly, 2005; Lee et al., 2016).

Hancock and O'Rielly (2005) encourage targeting preferably the C-terminus or the N-terminus of the protein as they are often more exposed in the structure and also advise avoiding high charged or hydrophobic regions.

Not all peptides are synthesized with equal effort. Some peptides might prove very difficult to synthesize. Hydrophilic amino acid sequences may be fairly simple to synthesize given that they are soluble. Peptide synthesis is encouraged to be handled by specialized companies providing custom peptide synthesis services. In-house automated

peptide synthesis equipment's cost can only be justified with extensive use of the machine. 5-20 mg of peptide is generally considered enough for antibody production (Hancock & O'Reilly, 2005; Lee et al., 2016).

Peptides themselves are too small to illicit an immune response. Therefore, they need to be covalently conjugated to a proven immunogenic carrier protein such as Bovine albumin serum (BSA) or Keyhole limpet hemocyanin (KLH). BSA is avoided if certain downstream applications are required while KLH has been reported to be similar to a plant protein to which anti-peptide Abs conjugated to KLH crossreact to when the Abs have been raised in rats (Oulehlova´ et al., 2009). Another approach is the multiple antigenic peptide (MAP) which is a scaffold that can support multiple peptides. The most standard conjugation is the glutaraldehyde method. It is highly recommended to purify anti-peptide by affinity chromatography to remove any none specific Abs (Hancock & O'Reilly, 2005; Lee et al., 2016).

e. Serological Tests for Phytoplasma

The detection of phytoplasma is still largely dependent on molecular methods with a notable increase in the rise of LAMP based field kits. Tomilson et al. (2010) reported that ELISA and lateral flow-base systems with the use of specific phytoplasma antibodies have largely been unsuccessful. A commercial serological detection system for apple proliferation phytoplasma is available but not deemed suitable for all applications. There have not been many reports of antibody-based phytoplasma detection phytoplasma antibodies since.

Many articles report successful polyclonal and monoclonal antibodies raised against purified phytoplasma or recombinant phytoplasma proteins. These antibodies were

developed for a range of applications such as localization/expression studies (Arashida et al., 2008), or specific detection (Hodgetts et al., 2014; Wambua et al., 2017). In the study by Arashida et al. (2008), the antibodies successfully and specifically detected the target phytoplasma in western blot tests, but the antibody was not tested as a diagnostic tool because that was not the scope of the article. Hodgetts et al. (2014) tested monoclonal antibodies raised against secA protein (not a membrane protein) and found them not suitable for routine detection. Wambua et al. (2017) is one of the rare examples of monoclonal antibodies raised against recombinant phytoplasma membrane proteins that have been tested and verified as viable specific and sensitive detection tools by ELISA and dot blot immunoassays (DBIA) and proposed to be applicable to lateral flow tests.

The lack of commercial universal or specific serological detection kits for phytoplasma is a major handicap to plant quarantine measures. Serological kits offer sensitivity and specificity comparable and sometimes better than qPCR, are simple and inexpensive, and can be applicable to field tests like LAMP tests. However, LAMP tests are prone to false-positive results and also require complicated primer design and are generally more costly than serological methods. Serological methods such as tissue blot immunoassay (TBIA) can prove useful for rapid disease detection and localization in the plant sieve tube elements, thus lowering the chance of false positives.

CHAPTER III
MATERIALS AND METHODS

A. Recombinant Membrane Protein Expression

1. Membrane Proteins

'*Ca.* P. phoenicium' integral membrane proteins were identified by Quaglino et al. (2015). Dr. Fabio Quaglino, University of Milan, kindly suggested 8 of these membrane proteins as suitable targets for immunogens. These proteins were designated F1 to F8 and the full sequence of their genes along with their names and suggested functions in the draft genome annotation are given in Appendix I, A.

Two additional proteins (Inmp and GroEl) that are commonly used as PCR targets for '*Ca.* P. phoenicium' were also tested as expression targets in Cell-Free expression system.

Based on several studies summarized in the literature review as well as the protocol described by Galetto et al. (2013) who reported difficulties in expressing full length phytoplasma membrane proteins and recommended solving this issue by expressing only the hydrophilic part of the protein, our 8 membrane protein sequences were analyzed by Protter 1.0 (Omasits et al., 2014). Examples of output are shown in Appendix I, D.

Out of the 8 membrane proteins, only F5, F7, and F8 were found to have non-transmembrane hydrophilic regions large enough (F7 somewhat smaller) for *E. coli* protein expression which becomes complicated with proteins smaller than 10 kDa. The respective regions were named 5N (13.8kDa), 7N (6 kDa), and 8N (11.37kDa) (Appendix I, A).

2. System Choice and Primer Design

For the expression of both the entire membrane proteins and the truncated regions, the T5 promoter based pQE-30 (Qiagen) /M15 competent cells (Qiagen), and the T7 promoter based pRSET A (Invitrogen) /C43 competent cells (Lucigen) systems were used. Both vectors contain a 6xHistidine tag upstream of the multiple cloning site (MCS). Primers were designed to amplify the full genes or the truncated regions of the genes. The primers included at their 5' ends sites of selected restriction enzymes and a few overhang bases to enhance restriction digestion of resulting amplicons and allow ligation into the MCS of the expression vectors used. Care was taken to choose restriction enzymes that do not have their target recognition site in the gene amplified. The primers are reported in Table 1 in Appendix I, B. and the maps of vectors used are reported in Appendix I, C.

3. Phytoplasma Source and Total Nucleic Acid Extraction

All AlmWB infected almond samples included in this study were collected from symptomatic trees in the Fghal and Smar Jbeil areas and their surroundings in the proximity of the city of Batroun. Total Nucleic acid extraction was carried out on 100 mg samples of phloem tissue with a cetyl-trimethyl ammonium bromide (CTAB) method. Samples were placed in 1.5 ml microcentrifuge tubes, and flash frozen in liquid nitrogen and ground with a microcentrifuge pestle and drill. 800 µl of 2% CTAB buffer (Appendix II, A) supplemented with 16 µl of β-mercaptoethanol was added onto each ground sample. These samples were incubated in a water bath at 60ºC for 20 min with mixing with vortex every 5 min. Subsequently, 600 µl of isoamyl alcohol-chloroform

(1:24 v/v) was added to the mixture, vigorously vortexed, and centrifuged at 10,000 rpm for 5 min in a Centrifuge 5804 R (Eppendorf). The supernatant was transferred to a clean microcentrifuge tube and 600 µl of ice-cold isopropanol were added. This mixture was kept at -20ºC for 30-60 min and later centrifuged at 14,000 rpm for 8 min. The supernatant was discarded, and the pellet was washed with 70% ethanol and centrifuged at 14,000 rpm for 5 min. The supernatant was discarded, and the ethanol was evaporated using a vacuum concentrator. The pellet was dissolved in 50 µl autoclaved distilled deionized water and stored at -20ºC.

Positive samples confirmed by AlmWB specific PCR with AW16sF/AW23sR primers as described by Jawhari et al. (2015) were used as DNA templates for PCR amplification of the targeted genes.

4. PCR

All PCR reactions were carried out in C1000 Touch™ Thermal Cycler (Bio-Rad). Each gene was amplified with its respective primers at an optimal annealing temperature as determined by running annealing temperature gradient runs. Reactions were carried out in 0.2 ml domed PCR tubes with a total reaction volume of 20 µl composed of : 12 µl of autoclaved distilled deionized water (ddiH_2O) , 4 µl of 5x FIREPol® Master Mix (Solis BioDyne), 1 µl of each 10 µM primer (final concentration 0.5 µM), and 2 µl of phytoplasma positive DNA template (Diluted to 10-50 ng/µl). Standard PCR protocols were run with the optimal annealing temperature for each set of primers and enough extension time (1 min/1 kbp) based on the length of the amplicon (Appendix I, A and B).

PCR products were verified by electrophoresis on 1% gels of Certified™ Molecular Biology Agarose (Bio-Rad) and TAE buffer diluted from 50x Tris/Acetic Acid/EDTA (TAE) (Bio-Rad). Gels were run in 1x TAE buffer in Wide Mini-Sub Cell GT Cells (Bio-Rad) powered by Power PAC 300 (Bio-Rad) at 100 V until dye front reaches end of the gel. BenchTop 1 kb DNA Ladder (Promega) was included as a ladder. Gels were stained in 5 µg/ml ethidium bromide solution for 15 min and rinsed with distilled water. Gels were visualized with UV Transilluminator 2000 (Bio-Rad) and photographed with Gel Doc XR+ system (Bio-Rad).

5. Cloning and Transformation

PCR products were purified with Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) following kit's instructions. DNA concentration was determined by NanoDrop™ 2000 spectrophotometer (Thermo Scientific). Amplicons were inserted into 50 ng of pGEM-T Easy vector (Promega) by TA cloning with a 1:3 vector to insert molar ratio using 0.5 µl of T4 DNA Ligase (5 U/µL) (Thermo Scientific) and its buffer in a total reaction volume of 20 µl, allowing fast ligation in only 10min at room temperature.

Different amounts of the ligation mixture (2-10 µl) were mixed on ice with 100 µl of $CaCl_2$ home-made XL1-Blue competent cells thawed on ice in 14ml polypropylene tubes. The mixture was left on ice for 30 min, heat shocked at 42ºC for 45 s and returned to ice for 2-3 min. 900 µl of Lysogeny broth (LB) were added to the mixture and incubated at 37ºC for 1 hour in a shaking incubator at 250 rpm. The cells were pelleted by centrifugation at 5000 rpm for 4 min and resuspended in 400 µl of LB. Aliquots of 100 µl and 200 µl were plated on LB agar supplemented with Ampicillin

(AMP) at 100 mg/ml and also with 100 µl of a solution containing 60 µl X-Gal (Appendix II, B), 30 µl LB+AMP and 10 µl of 0.5 M IPTG for blue-white screening of colonies. White colonies were propagated on LB+AMP and verified to contain the insert by PCR with M13 forward and reverse sequencing primers.

Plasmid purification from positive colonies was performed using Qiaprep® Spin Miniprep kit (Qiagen) following kit's instructions and sent for Sanger sequencing with the M13 forward and reverse sequencing primers at the "Unité de Génétique Médicale" of Saint Joseph University. Transformed bacterial cultures containing verified sequences were stored at -80ºC at a final 15% glycerol concentration.

6. Subcloning into Expression Vectors

All restriction enzymes were obtained from Thermo Scientific. The cloned inserts were digested out of pGEM-T easy vector by double digestion with the designed restriction enzymes and the buffer recommended by Thermo Scientific Double Digest Calculator. Restriction digestions were carried out in 50 µl reactions according to instructions of the enzyme manuals at 37ºC for 1 hour. The vectors were cut with the same enzymes as the inserts. The cut inserts and vector were separated on agarose gel and the bands were excised and purified with Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Ligation was carried out as described above for pGEM-T Easy (Promega) with the exception of using 100ng of the cut vector.

pQE-30+insert was transformed into homemade M15 competent cells, which have Kanamycin (KAN) resistance as a selection marker, as described above for cloning with the difference of culturing on LB+AMP+KAN plates rather than LB+AMP.

pRSET A+insert was transformed into C43 cells as described for cloning. In the case of pRSET A and C43 transformation was attempted on LB+AMP, LB+AMP+1%Glucose, and on M9 minimal media +AMP (Appendix II, C). For the pRSET A + C43 system, F4, F7, and 5N were tried. F1 was also attempted but failed to be transformed.

No blue-white screening was employed. Each of the colonies grown was propagated in liquid culture, tested by PCR for correct insert size and the purified plasmid was sequenced using sequencing primers specified by each vector manual as "Promoter Region" and "Reverse sequencing" for pQE vectors and "T7 promoter" and "T7 reverse priming site" for pRSET vectors. The sequences were carefully analyzed to verify that the correct sequence was inserted in frame with no modification upstream of the gene.

Homemade C43 competent cells were made following a simple $CaCl_2$ method and a more specialized protocol titled "Preparation of chemically competent C43 DE3" retrieved from protocolpedia (2018).

Transformation efficiency of $CaCl_2$ and new C43 cells made chemically competent in the lab was tested by transforming 20 pg of pUC19 vector into 50 µl of cells and plating on LB+AMP agar plates. The C43 strain was verified by transformation of 50 µl of lab made chemically competent C43 with 200 pg of pAVD10 containing a 17 kDa uncF gene coding for a toxic protein (ATP synthase subunit b) upon induction to BL21(DE3) and C41 cells and plating on LB+AMP with 1mM IPTG added to cells before plating them.

In some cases, digested PCR products were directly inserted into digested pRSET A expression vector and transformed into C43. For these, 60 µl of the new C43 cells were transformed with 110 ng of pRSET A and 1:4 molar ratio of vector to insert.

7. Protein Expression Screening

a. Induction

15 ml of broth (LB+AMP+KAN) for M15 and (LB+AMP, LB+Glucose+AMP or M9+AMP) for C43, were inoculated with 0.5 ml of overnight culture of the sequence verified transformed bacterial cultures. Cultures were incubated in shaking incubator at 37ºC and 250 rpm until optical density at 600 nm (OD_{600}) reached around 0.7-0.8. Inductions of cultures at OD_{600} of as low as 0.5 and as high as 1 were also tested.

Induction was performed by adding IPTG to the growth media at final concentration of 1 mM and 0.1 mM. Each was further grown while shaking at a combination of different conditions with temperatures tested being on ice, 20ºC, 30ºC, and 37ºC and incubation times ranging from 2 hours to 16 hours. Induction was also tested in one trial with M15 with the supplementation of 3% ethanol to increase expression level (Griffiths et al., 2000). The induced cells were centrifuged at 5000 rpm in a Centrifuge 5804 R (Eppendorf). The supernatant was discarded, and pellet was either frozen at -80ºC or immediately screened for expression.

b. Lysis, Solubilization, and Screening for Protein Expression

Different cell lysis buffers and methods were tried, but the one found most convenient and satisfactory was resuspension of the pellet in 1 ml Lysis Buffer (Appendix II, D) and lysis by sonication with MISONIX Sonicator® 3000probe

sonicator with MICROTIP™ attached. When no soluble protein of the expected size was detected, the pellets were resuspended in 8 M Urea buffer and sonicated (Appendix II, E) or 6 M Guanidine hydrochloride (GuHCl) buffer (Appendix II, F). The lysate was centrifuged at 15,000 rcf for 20 min and the supernatant containing all the soluble/solubilized proteins was transferred to a clean microcentrifuge tube.

10 µl samples of the supernatant and the solubilized pellet of expressed samples were mixed with equal volume of 2x SDS loading buffer (Appendix II, G) , incubated at 95ºC for 5 min and separated on 12% SDS-PAGE gels (Appendix II, H) for the larger proteins and up to 20% SDS-PAGE gels for the smaller proteins. Incubation at 37ºC for 1 hour in SDS loading buffer as recommended for membrane proteins was later employed. In the case of 6 M GuHCl, samples were diluted 1:4 with 8 M Urea Buffer to avoid GuHCl precipitation with SDS loading buffer. As soon as dye front entered the gel, the run was stopped and the wells were flushed with running buffer before continuing the run.

pQE-40 cloned in M15 expressing a 26 kDa DHFR protein was used as positive control for the M15 trials, and pAVD10 cloned in C43 expressing a17 kDa uncF protein was used as positive control for C43 trials. 7 µl of Precision Plus Protein™ Dual Color Standards (Bio-Rad) was run as ladder. The runs were carried out in Mini-PROTEAN® 3 Cell (Bio-Rad) with Tris-glycine running buffer (Appendix II, I) at 110 V until the dye front reached the end of the gel. Gels were stained with Coomassie blue staining solution (Appendix II, J) for 30 min and destained with destaining solution (Appendix II, K) until satisfactory amount of background color was removed.

Upon suspicion of a band of the correct size being expressed, standard protein purification was carried out with Ni-NTA Agarose (Qiagen) as recommended by the

supplier: the 6x-His tagged proteins were eluted either with varying imidazole concentrations or with pH gradient which both affect binding of 6x-His to Nickel beads.

B. Cell-Free Protein Expression

pRSET A plasmids with sequence verified His-tagged F4, F7, and 5N inserts cloned as described above were purified using QIAprep Spin Miniprep Kit (Qiagen) as described by the kit protocol. Another trial was conducted with the modification of not adding RNAse A in lysis buffer of the kit for fear that residual RNAse might interfere with transcription.

The purified plasmids were used as DNA templates in PURExpress®2.0 (CosmoBio) cell-free expression kit following the kit protocol. Liposomes were prepared from Soy Phosphatidylcholine and added to the reaction as described by Kuruma and Ueda (2015) to serve as a lipid membrane to which the expressed proteins can be inserted into. The reaction was carried out for 4 hours at 37ºC. 7 µl of 2x SDS loading buffer were added to 7 µl of the reaction and incubated at 37ºC for 1 hour (Cosmo Bio instructions for transmembrane proteins). The reaction mixtures were also incubated with detergents such as 8 M Urea and 1% Triton X-100 in an attempt to solubilize any possible insoluble expressed protein. A PCR template encoding an 18 kDa DHFR protein supplied with the PURExpress®2.0 (CosmoBio) kit was also used for expression as positive control. Aliquots of samples mentioned above were mixed with 2x SDS loading buffer and analyzed on 12% or 14% SDS-PAGE.

In a second trial, to avoid any complication that might be resulting from the use of plasmids, primers were designed as recommended by Kuruma and Ueda (2015) and the PURExpress® 2.0 kit manual for F7, inmp, and GroEl proteins (Appendix I, A and B).

A T7 promoter and a Ribosome Binding Site (RBS) were added upstream of the start codon using tagged primers with a nested PCR run. An AT-rich 6xHis tag and a glycine-serine linker were also added ahead of the protein gene. The amplicon bands were gel purified and used as DNA template. Protein expression of the three proteins was attempted without the addition of liposomes, as recommended in a personal communication with the Cosmo Bio team. After incubation for 4 hours, the reaction was run in SDS-PAGE as described above.

C. Anti-Peptide Antibodies

1. Peptide Design and Antibody Production

The Sequences of our 8 membrane proteins were analyzed by Protter as described in step A.1. Non-transmembrane regions of these sequences which are 15-20 amino acids (aa) long (optimal recommended length) were analyzed using Predicting Antigenic Peptides (Immunomedicine group, 2018) software. Possible antigenic peptides were analyzed for specificity to '*Ca.* P. phoenicium' with blastn. A list of 15-20 aa long, specific, antigenic, and non-transmembrane peptides were sent to multiple companies to provide quotations for peptide synthesis. It was found more economical to purchase affinity-purified polyclonal antibodies raised against these synthesized peptides from GenScript Biotech Corporation (Hong Kong). Upon discussion with their specialists, custom packages were designed for us for affinity purified polyclonal antibodies raised in rabbits against synthesized F2, F5, and F8 peptides (Appendix I, E).

2. Serological Tests

Young green shoots around 3 mm in diameter were sampled from symptomatic AlmWB infected almond trees in the Feghal and Smar Jbeil area. PCR-negative shoots as healthy control were gathered from almond trees in AUB AREC campus. For Tissue Blot Immunoassay (TBIA), 2 mm thick cross sections of the stem were cut and pressed gently onto 0.2 µm nitrocellulose Trans-Blot® Transfer Medium (Bio-Rad) for 10-15 s. 2 µl drops of synthesized peptides were used as positive control. Nylon membranes were also tested but eliminated due to high background.

For a subsequent test, shoot and petiole samples from various healthy stone fruit trees(cherry, plum, peach, apricot, and almond) from an irrigated orchard in Hammana were tested in TBIA with anti-F8 Abs. On the same membrane tissue prints from healthy almond trees from South Lebanon and from Bekaa, and infected samples from Thoum region were also blotted.

Membranes were washed once by TBS and then blocked by 5% skimmed milk and 1% BSA in TBS buffer (Appendix II, L) for 1 hour while shaking. Incubation with 0.5% Polyvinyl alcohol for 1 hour was also tried as a blocking solution with similar results. Membranes were washed twice while shaking for 5 min in TBST. Lyophilized primary antibodies anti-F2, anti-F5, and anti-F8 obtained from Genescript were solubilized in TBST with 1% BSA at 10 µg/ml. Blocked membranes were incubated with each of the primary antibodies or combinations of two of the antibodies at 37ºC while shaking. After three 5 min washes with TBST, membranes were incubated with 1:20,000 diluted Anti-Rabbit IgG (whole molecule) - Alkaline Phosphatase antibody produced in goat (Sigma-Aldrich) at 37ºC while shaking for 1 hour. Membrane was washed 3 times with TBST for 5 min.

Colorimetric detection was performed using the NBT/BCIP-based Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad) following kit instructions. Color development was stopped by washing the membrane with distilled water.

Dot Blot Immunoassays (DBIA) were performed with 2-5 µl drops of phloem protein extracted by grinding phloem tissue in PBS (Appendix II, M). Further steps were as described for TBIA. Detection was attempted both with NBT/BCIP colorimetric method and CDP-star® chemiluminescense method, noting that the substrate used was past its expiry date.

In an attempt to reveal the size of the protein to which the antibody might be reacting with, Western Blot was performed with anti-F8 antibodies. Infected almond stem phloem was ground in western blot extraction buffer (Appendix II, N) and separated on 12% SDS-PAGE. The proteins on the gel were transferred to nitrocellulose membrane at 60 V for 1 hour in Western Blot transfer buffer (Appendix II, O). Transferred proteins on nitrocellulose membranes were processed as described for TBIA.

CHAPTER IV
RESULTS AND DISCUSSION

A. Protein Expression in E. coli

1. PCR for Amplification of Membrane Protein Genes

All the PCR primers designed for each membrane protein (8 original proteins and 3 truncated ones) successfully amplified the target protein from AlmWB symptomatic almond tree DNA extracts with the unique correct sized band visualized on 1% agarose gels. These desired results were reached after optimization of annealing temperature by running temperature gradient PCR runs for each of the primer pairs (Fig. 1). No band was observed in the negative controls run with healthy almond DNA extracts.

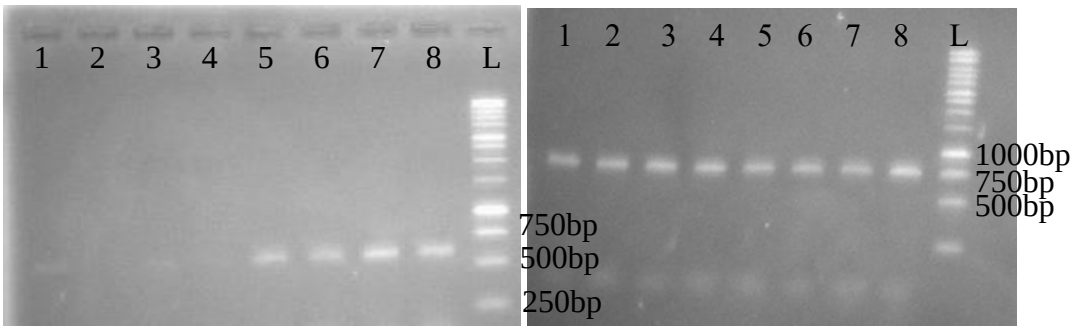


Figure 1: Agarose gel electrophoresis of PCR amplicons from AlmWB infected samples using (A) F5/R5 primers.; 1-8 = 60-57°C annealing temperature gradient: 580 bp F5 amplicon and (B) F6/R6 primers. 1-8 = 51-48°C annealing temperature gradient: 717 bp F6 amplicon. L= 1 Kbp ladder

2. Cloning into pGEM-T Easy Vector

PCR products were inserted into pGEM-T Easy vector and transformed into XL1 Blue competent cells for blue-white colony screening (Fig. 2). Through repeated

trials all membrane proteins were cloned into pGEM-T Easy. Miniprep and PCR of white colonies confirmed correct band size and sequencing confirmed the integrity of each of the targeted nucleotide sequences (8 full gene sequences and 3 truncated gene sequences) inside the vector (Fig. 3).

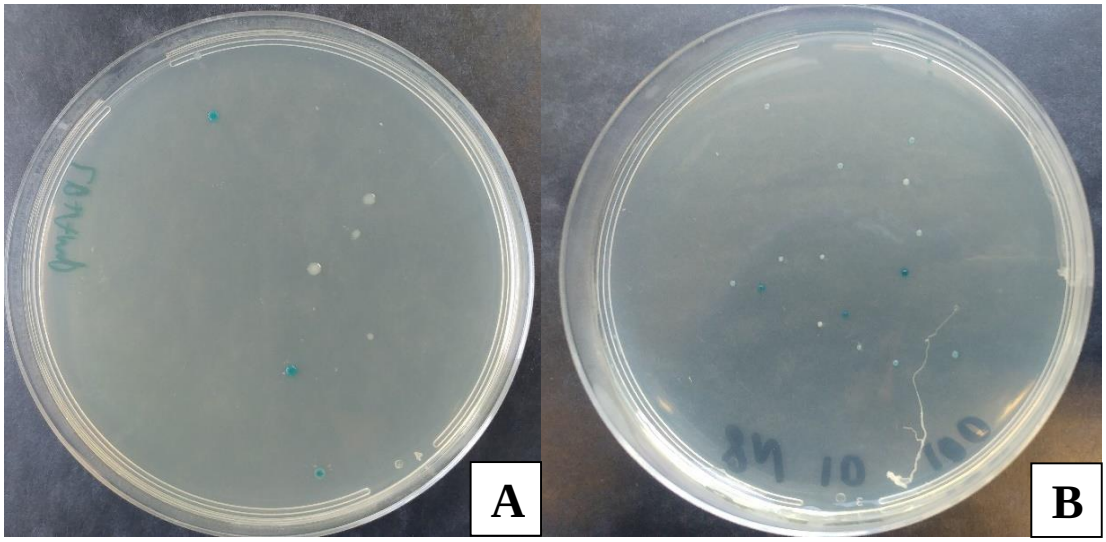


Figure 2: White and blue colonies of transformed XL1 blue competent cells carrying pGEM-T Easy inserted with (A) an intact membrane protein and (B) a truncated membrane protein on IPTG and X-Gal coated LB agar plates.

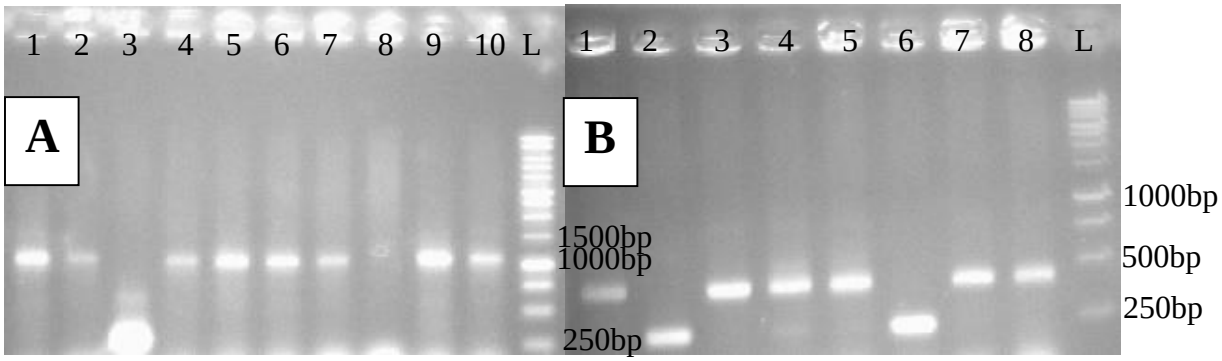


Figure 3: Agarose gel electrophoresis of PCR products from colonies (A) transformed with F7(844 bp) inserted in pGEM-T Easy using M13 Forward and Reverse sequencing primers of pGEM-T Easy which add approximately 200 bp to inert size. Colonies in lanes 1, 2, 4, 5, 6, 7, 9, and 10 possess the correct size of around 1050 bp (844+200 bp). The colony in lane 3 represents a self-ligated vector possessing a 200bp indicative of the size of only M13 amplicon with no insert. (B) Transformed with the truncated 7N (169 bp) in pGEM-T Easy with colonies in lanes 1, 3, 4, 5, 7, and 8 indicating the correct band size of pGEM-T Easy+7N (369 bp) and colonies in lane 2 and 6 represent self-ligated vector with the 200 bp size. L= 1 kbp ladder.

3. Sub-cloning into Expression Vectors

Purified pGEM+insert of all proteins and/or direct PCR products (when attempting to directly insert PCR product into expression vector) along with the recipient vectors were successfully digested by restriction digestion as shown by the presence of two bands corresponding to the exact sizes of the linearized pGEM vector and the insert (pGEM+insert) (Fig. 4). No larger or smaller bands representing circular or supercoiled plasmid whose sizes are skewed from the actual bp content were observed. This is also the case for the recipient vector representing only one band of the cut linear plasmid at the correct size (Fig. 4).

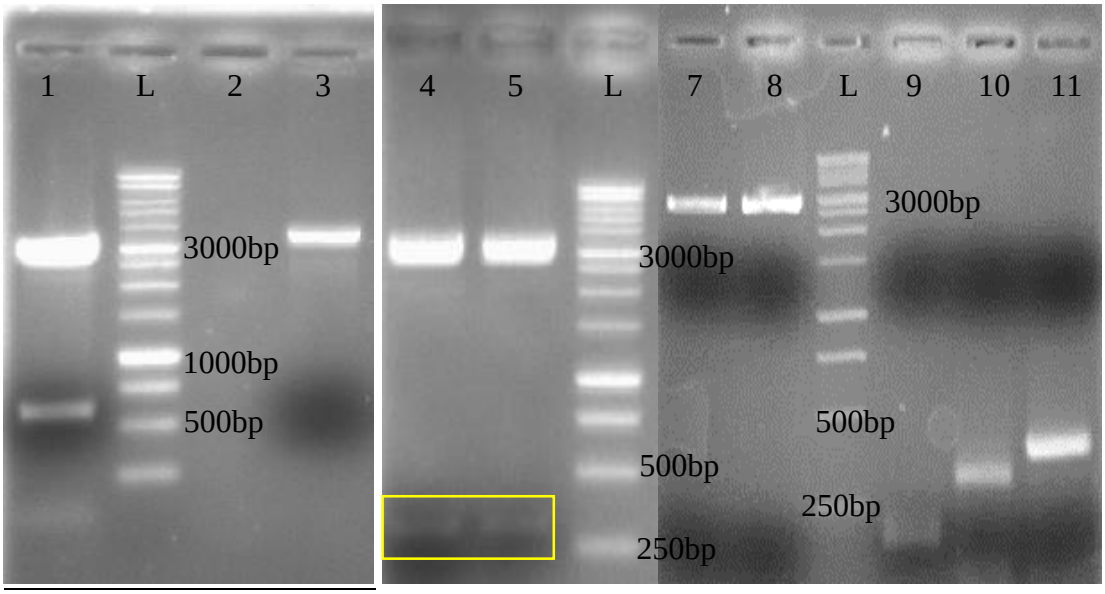


Figure 4: Agarose gel electrophoresis of restriction digestion products of (Lane 1) pGEM+F5 with bands corresponding to F5 (564bp) and linear pGEM-T Easy (3015 bp) but no band for undigested pGEM+F5 (3600 bp), and (Lane 3) linearized pQE-30 (3461 bp). Lanes 4 and 5 display digested pGEM-T Easy+8N with the linearized pGEM-T Easy (3015bp) and the cut insert 8N (285 bp) within by the yellow box in the figure. Lanes 7 and 8 represent linearized pRSET A (2900 bp) and lanes 9, 10, and 11 represent digested 7N (153bp), 8N (285 bp), and 5N (354 bp) truncated MP gene PCR amplicons, respectively. L= 1 kbp ladder.

Subcloning into the two expression vectors (pQE30 and pRSET A) was difficult to achieve for the membrane proteins and was only successful into pQE-30 when transformed into M15 after several trials (Fig. 5).

For pRSET A, when using the chemically competent C43 the company supplied or when using homemade competent cells made with a simple $CaCl_2$ method, F1 failed to be subcloned after numerous trials. F4 and F7 were successfully subcloned only after numerous trials and their sequences were verified, but the transformation efficiency was incredibly low as shown in figure 5 with only a single colony per 2 plates for F4. Switching to M9 minimal media in one case (Appendx II, C) increased transformation efficiency significantly where 10-15 colonies were obtained per plate in comparison to

no colonies or a maximum of 1-2 on LB media. This trial however was not repeated to confirm the findings.

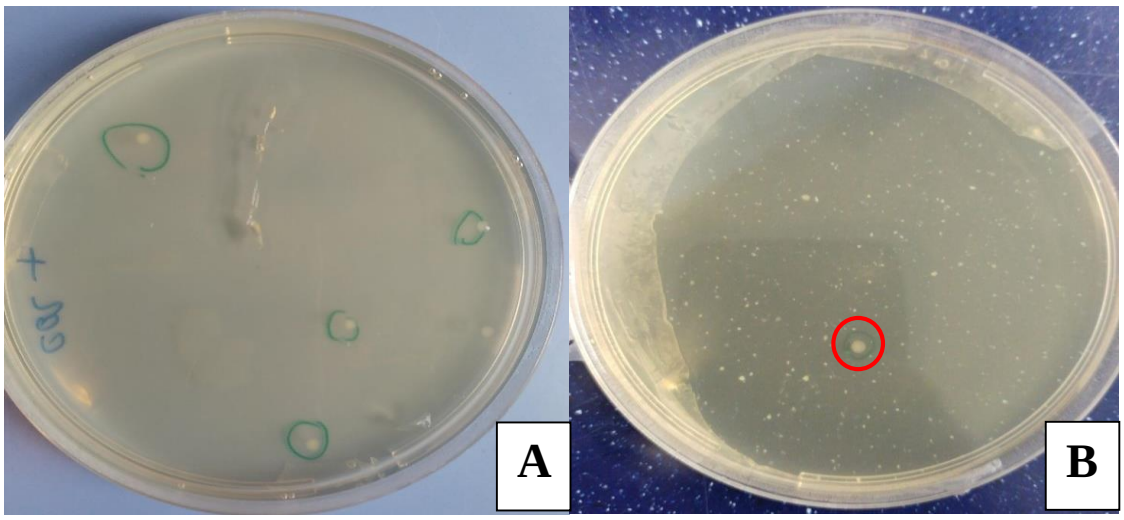


Figure 5: Transformed colonies of one of (A) the truncated membrane proteins inserted in pQE-30 vectors in M15 cells and (B) a single colony of F4 inserted into pRSET A vector in C43 cells.

In one case, the sequenced insert from a single colony obtained was revealed to be incorrectly cut upstream of the gene after the start codon at a sequence similar to the target sequence of BamHI resulting probably from star activity (although that specific sequence has not been reported in literature for BamHI star activity). This incorrect cut altered the frame of the gene and resulted in a stop codon which might explain the survival of that colony.

Upon failure of transforming control plasmid pUC19, a new batch of chemically competent C43 were made following the protocol from protocolpedia (2018). These C43 cells transformed with pUC19 control vector exhibited approximately 6 x 10^6 cfu/µg pUC19 transformation efficiency on LB medium (Fig.6). The competent cell

strain was verified to be C43 with the pAVD10 plasmid successfully grown on LB+AMP plates supplemented with 1 mM IPTG (Fig.6).

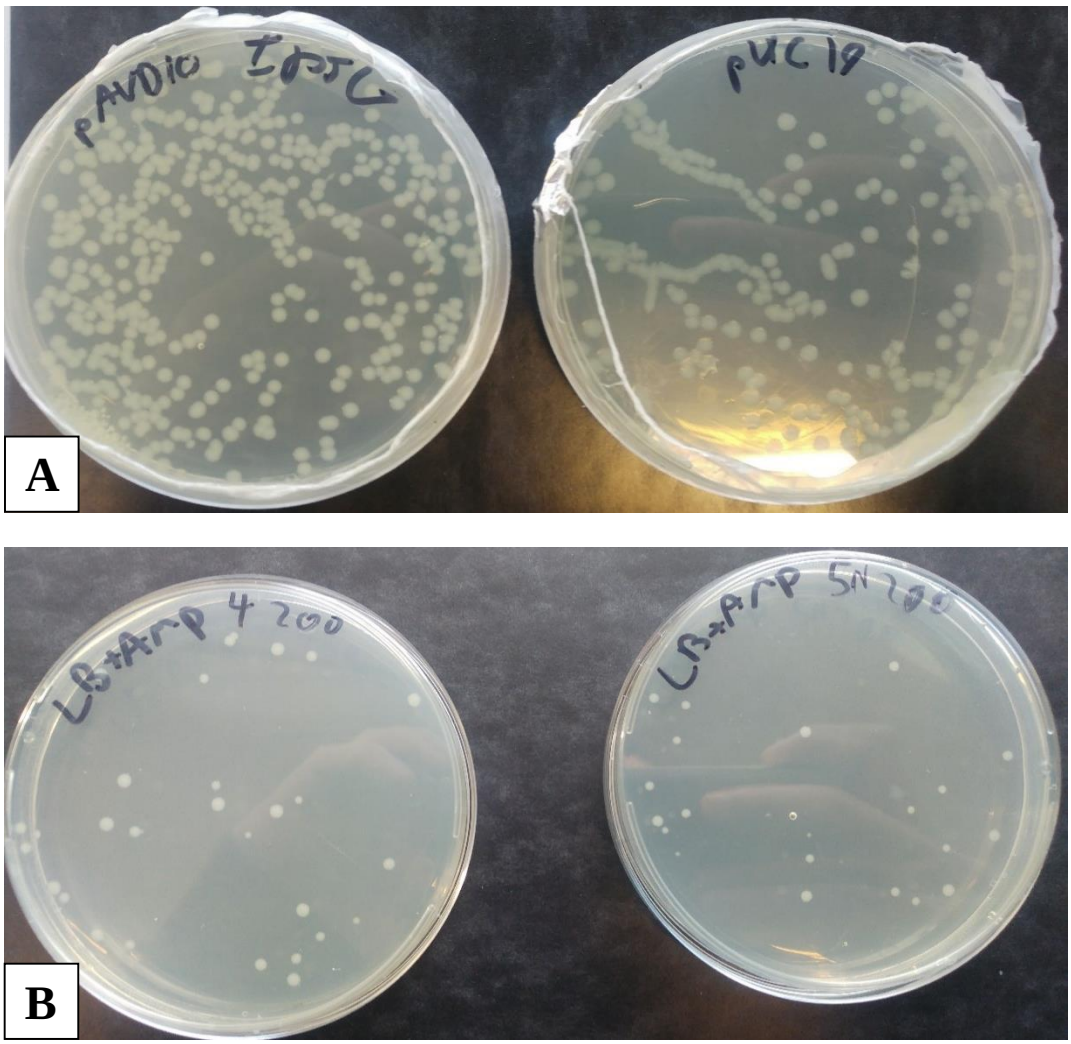


Figure 6: (A) LB+AMP agar plates with C43 *E.coli* colonies containing strain verification plasmid pAVD10 (with IPTG) and transformation efficiency control plasmid pUC19. (B) C43 colonies containing F4+pRSET A and 5N+pRSET A.

As seen in Figure 6 (B), transformation with pRSET A+F4 and pRSET A+5N had highly satisfactory efficiencies. This rules out previous suspicions of the protein being toxic to the C43 at basal level expression that was hypothesized to be the cause of

the low transformation efficiency of the old batch of C43. Transformation was tried in parallel with LB and M9 media with the new batch of cells and transformation efficiencies were similarly satisfactory. However, colonies appeared around 17 hours on LB agar while it took 24+ hours on M9 agar.

4. Screening for Protein Expression

Following SDS-PAGE analysis of the cultures of transformed bacterial colonies containing verified sequences of the inserts (8 membrane protein genes and 3 truncated genes) that were induced for protein expression, no signs of target protein expression was observed in total proteins, soluble lysates, insoluble lysates dissolved in detergents, or Ni-NTA purified (in the case of suspected bands of correct size). However, the control protein was successfully expressed, and the expressed control protein band was much more pronounced upon induction with 3% ethanol which is in line with the findings reported by Chhetri et al. (2015) (Fig. 7). This ethanol method to enhance expression was tested in case our target protein was suspected of being expressed but at a very low undetectable level. Thus, if this method increased expression levels we would be able to detect it. No additional protein bands of the expected size corresponding to the cloned proteins were detected upon comparison between ethanol induced and regular induced protein by SDS-PAGE analyses.

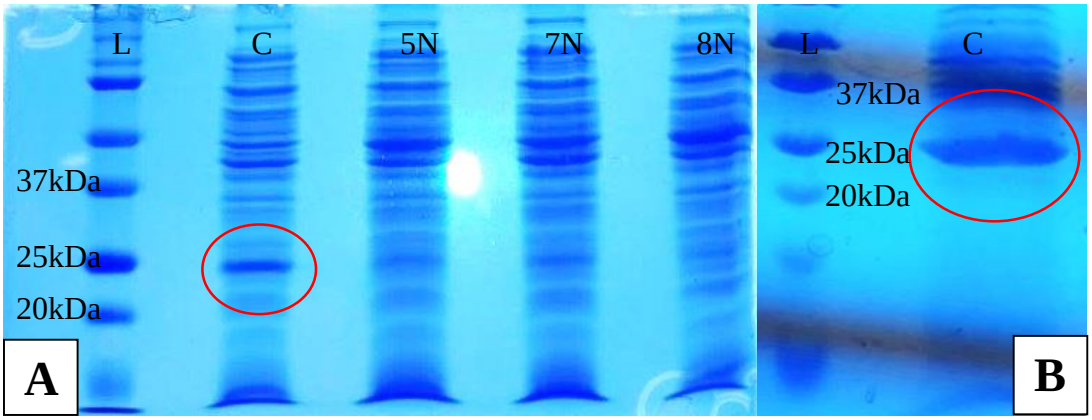


Figure 7: SDS-PAGE gels of total lysate of (A) the control protein, 5N, 7N, and 8N and (B) the control protein with 3% ethanol induction. L= Ladder.

No target protein was observed in all of the full and truncated proteins (total 11 proteins) tested in the pQE30+M15 system. Trials to optimize expression conditions such as induction time, temperature, and IPTG concentration were to no avail.

In the pRSET+C43 tests, only F1, F4, F7, and 5N were tried because of the compatibility of restriction sites applicable to these genes and to the MCS of pRSET A and also due to the already enormous amount of time, resources and effort put into *E. coli* expression. Hopes of the possibility of another protein being successfully expressed was not a very promising goal to pursue any longer.

Only F4, F7, and 5N were successfully cloned into pRSET A and transformed in C43. For both, no distinct protein band of the expected size was observed upon IPTG induction. Whenever a faint band was suspected Ni-NTA purification was performed to purify our 6x-His tagged protein. Figure 8 is an example of Ni-NTA purification of total lysates of F4. The band encircled in red was suspected to include our 21 kDa protein even though it is around 25 kDa because membrane protein sizes on SDS-PAGE gels do not always correlate well with their formula molecular weight (Rath et al., 2009).

However, Ni-NTA purification did not reveal the presence of histidine tagged protein band thus putting that argument to rest.

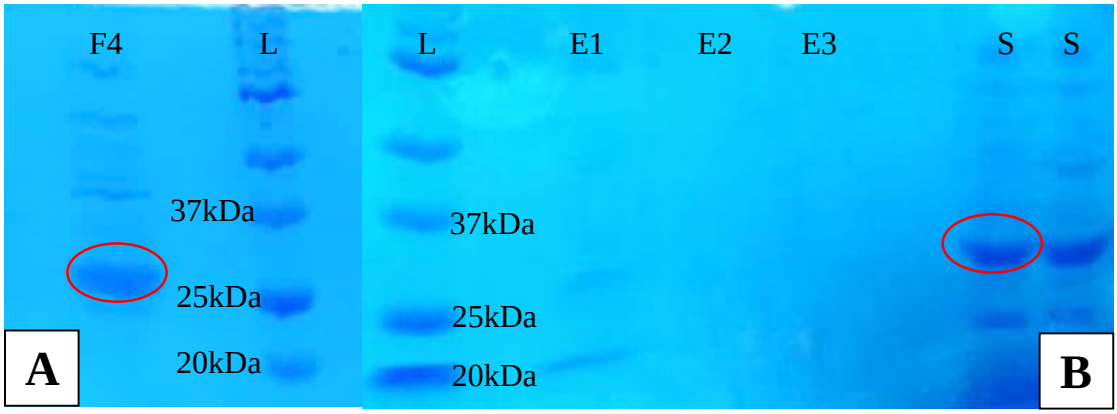


Figure 8: SDS-PAGE analysis of total lysate of (A): F4 cloned into pRSET A in C43 cells with suspected expression within a thick band in around the size of target protein, and (B): Ni-NTA purification of lysate revealing no bands in elutions (E1, E2, E3) and the same band in the supernatant (S) . L=Ladder.

For the increased number of F4 colonies on M9 minimal media, all the colonies screened for expression showed no target protein band (Fig. 9). In all trials, after cell lysis the soluble supernatant was analyzed, as well as the pellet dissolved in 8 M Urea. In some later trials, other detergents were also tested such as Triton X-100. None of the treatments performed to dissolve the insoluble proteins resulted in appearance of the target membrane protein band, eliminating the possibility that our target protein was expressed in a soluble or insoluble form.

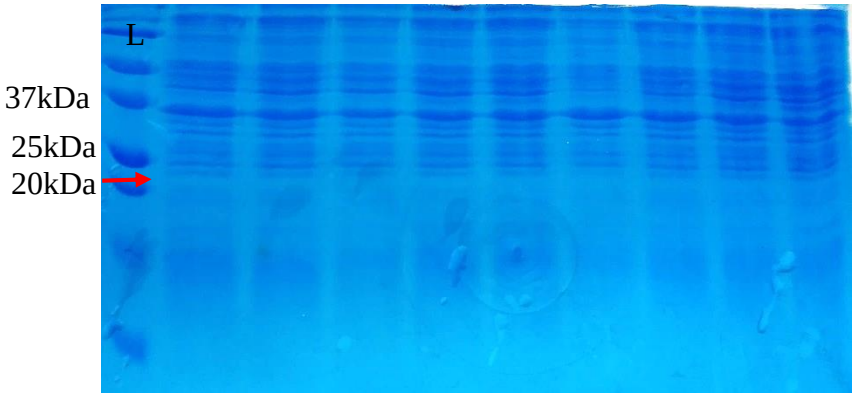


Figure 9: SDS-PAGE screening of 12 colonies (8 in figure) of F4 inserted in pRSET A transformed into C43 cells with no significant expression of a protein band around the expected target protein size represented by the red arrow (21 kDa). L=Ladder.

With the colonies obtained with the new batch of C43. Five colonies of F4 and five colonies of 5N were screened for protein expression with lysis in 8 M Urea Buffer and 6 M GuHCl buffer + 0.5% Triton X-100 (Figure 10). As observed in Figure 10, the control ATP synthase subunit b protein (which is an *E. coli* membrane protein) in strongly expressed while no band can be seen for our target proteins.

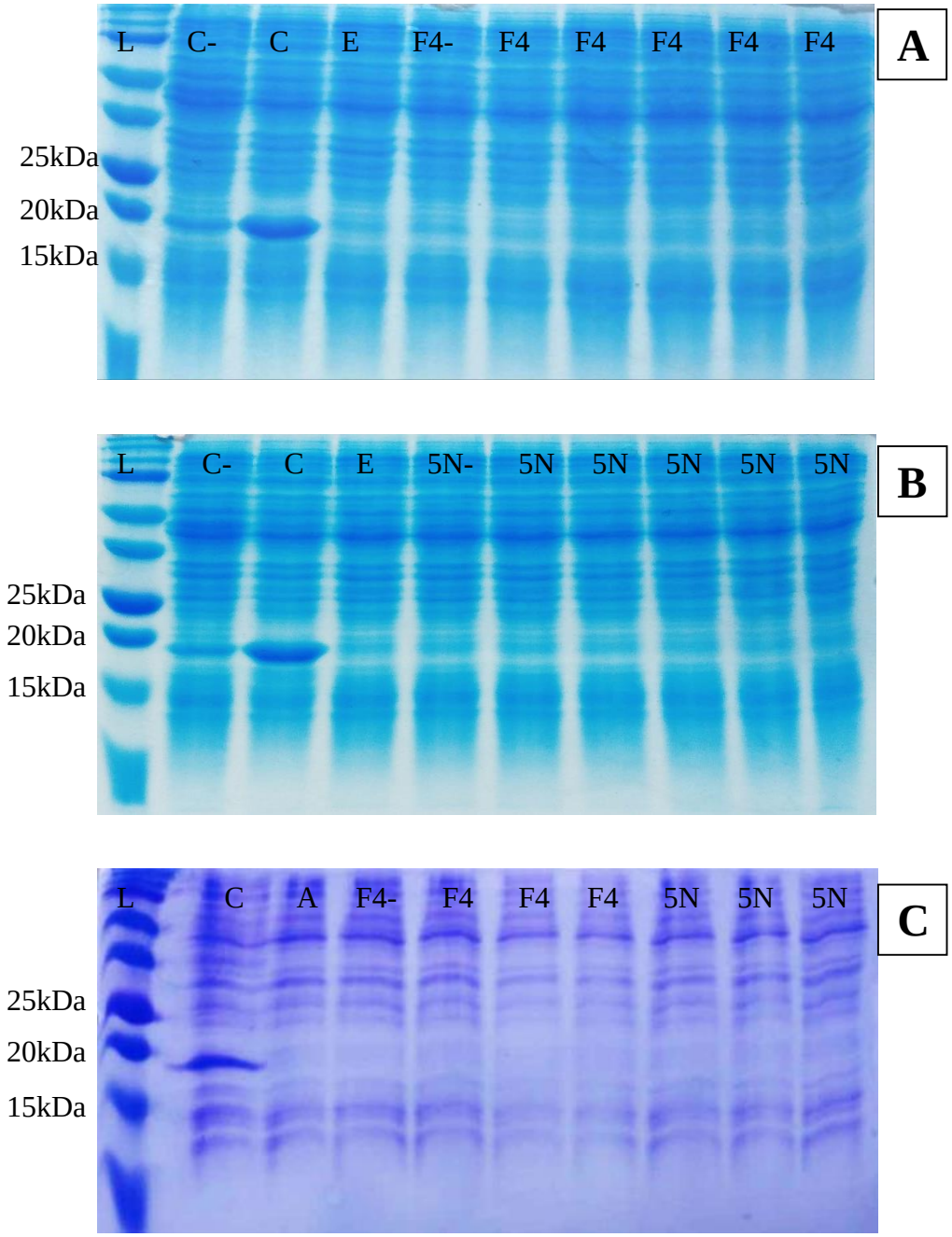


Figure 10: 14% SDS-PAGE analysis of (A): F4 lysates in 8M Urea buffer, (B): 5N Lysates in 8 M Urea buffer, and (C): F4 and 5N lysates in 6 M GuHCl buffer+ 0.5% Triton X-100. C= control protein. A= Empty induced pRSET A. C- = uninduced control protein. F4- = uninduced F4. 5N- = uninduced 5N. F4 and 5N = induced lysates of different colonies. L=Ladder.

B. Cell-Free Protein Expression

No band was obtained in SDS-PAGE gel corresponding to the target protein size for F4 , F7, or 5N, but a clear positive control band was obtained (Fig. 11). Solubilization with 8M Urea or 1% Triton X-100 for possible insoluble proteins also did not yield any detectable protein expression. The addition of liposomes (Fig. 12) in the reaction mixture also did not produce any detectable difference.

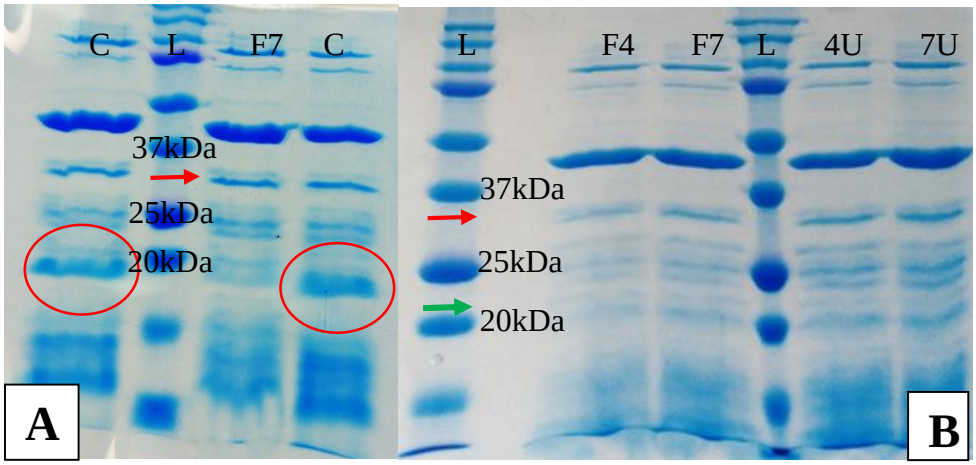


Figure 11: SDS-PAGE gels of cell-free expression products. (A): Clear 18 kDa band of control DHFR protein circled by red can be seen in the control C, while no band of the expected size for F7 (36 kDa) indicated by the red arrow is observed. (B): represents the total reaction products of F4 and F7 and the 8 M urea solubilized products 4U and 7U. The red arrow marks the expected size of F7 and the green arrow marks the expected size of F4 (21 kDa). L= ladder.

The presence of a unique protein band of the correct size for the control protein eliminates any errors with the reaction setup and conditions, as well as the integrity of the kit. The manufacturers and the selected publications such as the protocol reported by Ueda and Kuruma (2015) that we followed have worked with PCR products as DNA templates for expression in this system, they both reported plasmid DNA being valid

and applicable, but was avoided due to the extra time period it would take to prepare and verify the plasmid. At start, we chose to work with plasmids rather than PCR products because we had T7-promoter based pRSET A plasmids ready and sequence verified for F4 and F7 proteins from the *E. coli* expression trials. Also, we wanted to avoid the additional expenses and expected long delivery time to ship the new primers to Lebanon, especially in the case of the long specialized T7 promoter and ribosome binding site (RBS) sequence tagged primers required to produce a suitable template for the PURExpress®2.0 system. Scott et al. (2016) have successfully used a plasmid as a DNA template, but most papers that have used this new kit or its predecessor PURExpress® 1.0 have used PCR templates (Furusato et al., 2018; Niwa et al., 2015) or mRNA templates (Fan et al., 2017). It should be pointed out that the plasmids successfully used by Scot et al. (2016) were provided by Dr. Kuruma, who is one of the scientists who developed the PURExpress®2.0 system.

Figure 12: Final dried liposome powder of acetone washed Soy-PC.

Residual RNAse A from the miniprep kit was suspected to be interfering with the in-vitro protein expression, so it was eliminated from the miniprep reagents, but no change was observed.

Either pRSET A, for some reason, is not a suitable plasmid for protein expression in this system (for no apparent reason we can think of), or there is some inhibitor or contaminant involved in our DNA template (although the manufacturers recommended the Qiagen miniprep plasmid purification kit we were using), or most likely, the *E. coli* based system is incapable of synthesizing the targeted phytoplasma membrane proteins.

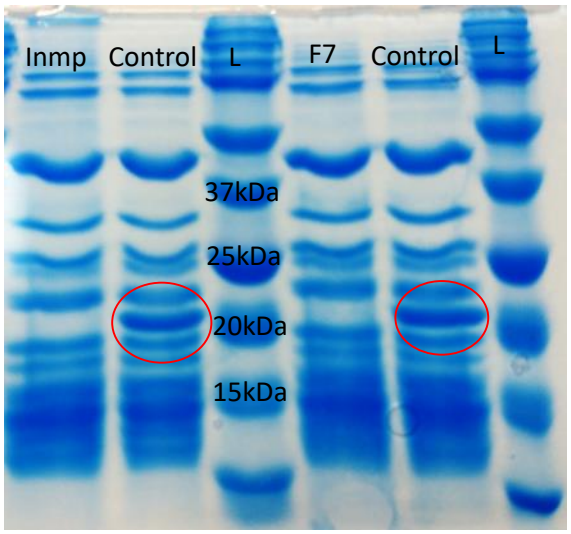


Figure 13: SDS-PAGE gels of cell-free expression products. No unique or overexpressed bands are observed for the inmp (23 kDa) or the F7 (29 kDa) membrane proteins. The control DHFR protein (18 kDa), circled in red, is clearly strongly expressed. L = Ladder

At a later stage, the failure of expression of the targeted proteins using plasmids, prompted us to order PCR primers that allow protein expression from PCR products in

cell-free systems. The tests with PCR product templates were also negative (Fig. 13) suggesting that the proteins we have chosen are not suitable for translation with *E. coli* machinery, because there are no more possible interfering factors. By using the cell-free system, we eliminated the possibility of the target protein being toxic to the *E. coli* cells. However, the system still utilizes *E.coli* based recombinant cell machinery, which might not be suited to synthesize these integral membrane proteins. A possible way forward for *E. coli* or *E. coli* based expression would be codon optimization based on the codons preferred by *E. coli* , or alternatively, trying other cell-free systems such as ones based on plant expression machinery.

C. Anti-Peptide Antibodies

The DBIA tests with all three antibodies were negative with NBT/BCIP assays with all the positive peptide controls staining strongly (Fig. 14). The strong background is due to anti-F5 antibody that kept exhibiting high background regardless of membrane and blocking method.

Anti-F8 was also tested in DBIA with no background but same negative results (results not shown). To expect good results with this amount of extract was very optimistic considering the low titer of phytoplasma in plant tissue (as low as usually requiring nested PCR for detection). In general, I would not regard DBIA as a good method for phytoplasma detection from regular plant extracts especially with NBT/BCIP staining. Regardless of the amount of extract passed through the membrane, the huge amount of plant proteins binding will inevitably overwhelm any small amount of the target phytoplasma protein.

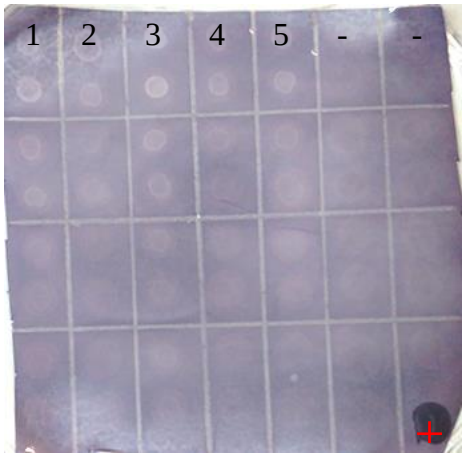


Figure 14: DBIA results of extract blots with columns 1-5 representing 5 different symptomatic infected almond trees, - representing healthy almond trees, and + representing the peptide positive control, tested with anti-F5 antibodies

The first antibody tested for TBIA was the anti-F5. The results with this antibody were unsatisfactory due to the high background color (Fig. 16) and no characteristic staining in sites corresponding to the phloem tissue as should be observed with phloem-limited pathogens (Ding et al., 2015). This high background can be attributed to the anti-F5 antibody because it was not observed with anti-F8 and was much less with anti-F2 (Fig.16) regardless of blocking with BSA + skimmed milk or with polyvinyl alcohol. In TBIA with anti-F5, a few positive samples exhibited peculiar staining patterns suspected of being indicative of phloem-limited phytoplasma. However, this hypothesis was rapidly discarded due to the presence of similar staining patterns in some negative controls, as well as due to the very low percentage of the positive samples that exhibited this staining (Fig. 15). Anti-F2 failed to react to any sample.

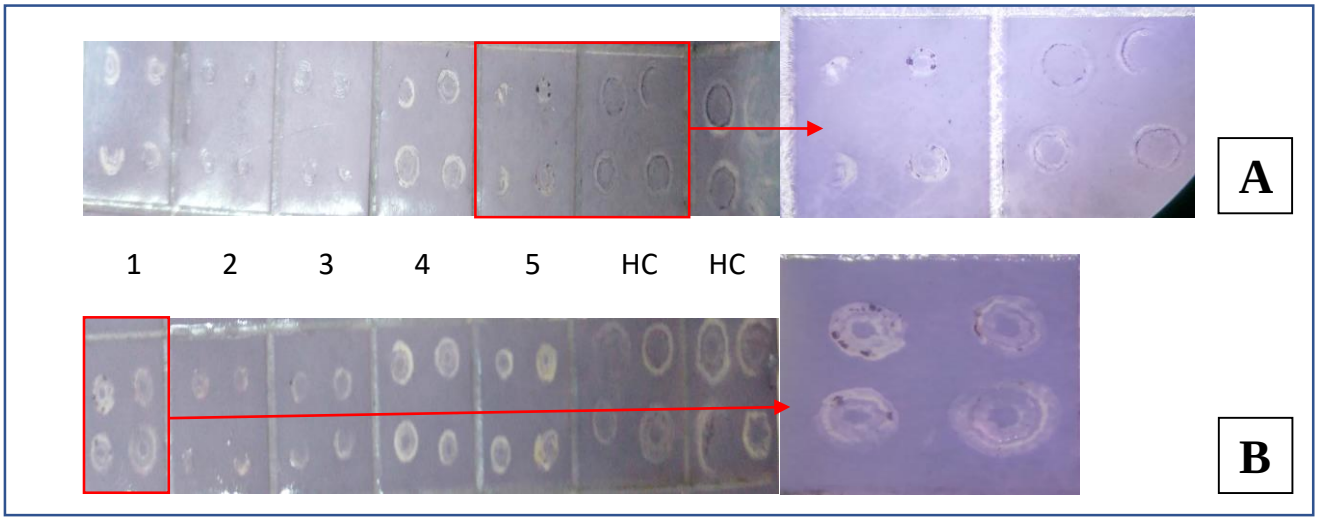


Figure 15: Two replicates of TBIA with anti-F5 antibodies. Lanes 1-5 representing samples from symptomatic infected almond trees and HC representing samples from healthy almond trees. In (A), only shoots of the column 5 tree of the infected samples showed staining as seen in the magnified image on the right, but the samples next to it in the – lane show staining in ring pattern. In (B) also only infected samples of the column 1 tree show peculiar staining as (A) which is not quite in a ring pattern but located around the vascular tissue.

We are inclined to believe the stains seen in anti-F5 tests are results of blotting membrane injury. In any case, our failure to replicate them renders them insignificant. The failure of anti-F5 and anti-F2 was no surprise as anti-peptide antibodies have a low chance of success. This may be attributed to the antibody epitope of the linear peptide not cross reacting with the native protein sequence as a result of protein folding rendering this peptide inaccessible within the protein structure. Even the 50% chance of success mentioned in Chapter II is somewhat optimistic considering that most advanced tools of membrane protein folding and membrane insertion prediction rely on databases of proteins from organisms that have been heavily studied, but not usually related to phytoplasma proteins.

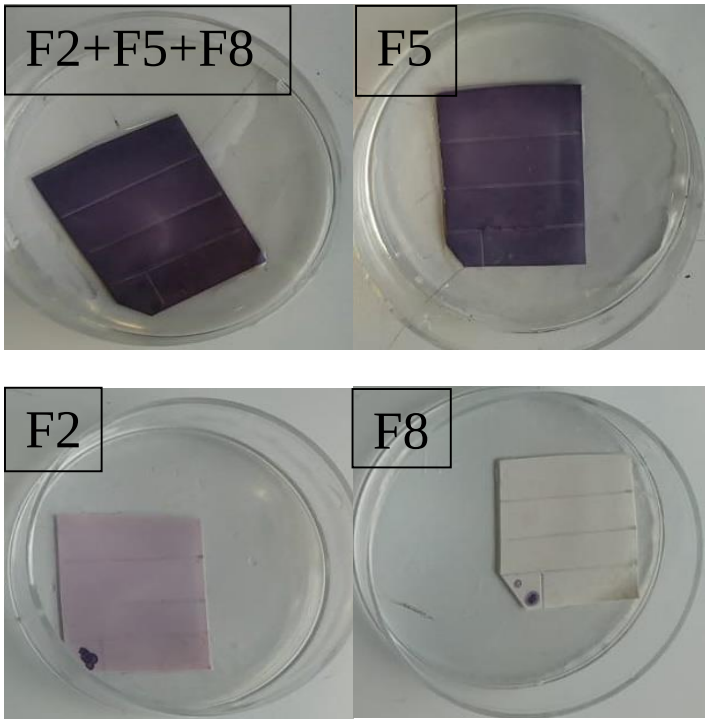


Figure 16: A comparison of the background of the three different antibodies and all of them combined.

The anti-F8 antibody reacted in an ideal dotted ring pattern characteristic of phloem-limited bacteria in shoots (Ding et al., 2015) with the infected tree samples. However, the appearance of similar staining in nested PCR negative healthy tree samples was also observed in Figure 17 and was much more pronounced in Figure 18.B. These results were replicated several times. These patterns were also observed, although much less pronounced, in non-AlmWB susceptible plants tested such as cherry and plum with a very strong pattern observed for healthy almond samples (Fig. 18). The initially observed positive results are ideal and essentially our intended objective. However, the similar pattern detected in healthy almond and non-host plants indicates that the antibody is not cross reacting with phytoplasma but some other protein(s) present even in healthy trees refuting our breakthrough. We have multiple possible explanations for the observed phenomenon ordered from most likely to least likely:

(i) The antibodies are reacting with a phloem protein, which would also explain the added thickness in healthy blots which have a much more robust vascular system than diseased tissue. However, no plant proteins were matched when our peptide sequence was blasted using blastp in the 'Prunus *dulcis*' organism database, or any other plant protein for that matter. It could be claimed it is an unrecorded one, but that is highly unlikely. The use of KLH as a conjugate in the peptide-KLH immunogen in rats has been recorded to result in anti-KLH antibodies which react with plant proteins by Oulehlová et al. (2009) which were reported to persist even after removal of anti-KLH antibodies by purification over a KLH affinity column. Our antibodies raised in rabbits were affinity purified with the antigen peptide. The possibility remains, that it is not our anti-peptide antibody (which did not show any blastp similar plant proteins) but instead the anti-KLH antibodies that might be causing the reactions against plant proteins. What weakens this argument , however, is the absence of such a reaction with the anti-F2 and anti-F5 antibodies which were also conjugated to KLH and raised in rabbits.

(ii) The antibodies are in fact reacting with phytoplasma. But the titer of phytoplasma is too low in the apparently healthy, but infected symptomless plants to be detected by PCR. This argument is based on the observed phenomenon of plants becoming infected by '*Candidatus* Phytoplasma phoenicium' (confirmed by nested PCR) but remaining essentially healthy and symptomless. This is unlikely to be the case because nested PCR is a very sensitive detection tool.

(iii) The antibodies are reacting with a phloem symbiont microorganism. Which is just wishful thinking with no evidence to back it up.

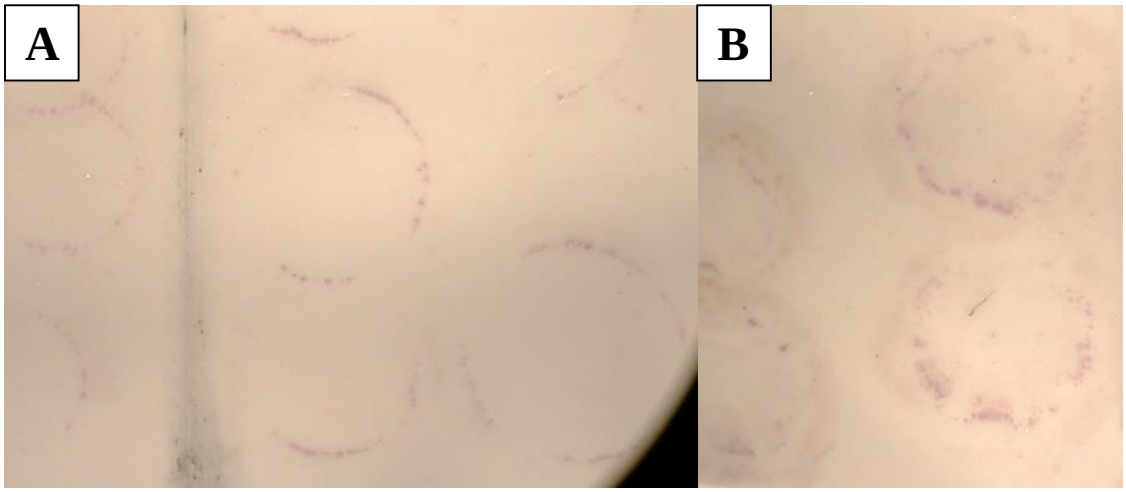


Figure 17: TBIA results: (A) The phloem-limited bacteria characteristic dotted ring pattern staining in infected tree tissue blots, and (B) a similar and arguably more pronounced pattern observed in healthy tree samples.

These tests were all carried out in the summertime of a very dry year. Some even towards the end of the summer because of the time of delivery of the antibodies. Most infected samples were collected from non-irrigated trees. So, the general condition of the sampled shoots was not ideal and phytoplasma titer must have been down. This might also explain the more pronounced staining in healthy almond samples in Figure 18.B. These tests will be repeated in springtime when phytoplasma titers will be higher. This might prove to be a deciding factor for anti-F2 and anti-F5 which did not react with infected samples in the summer.

A) Hammana Orchard

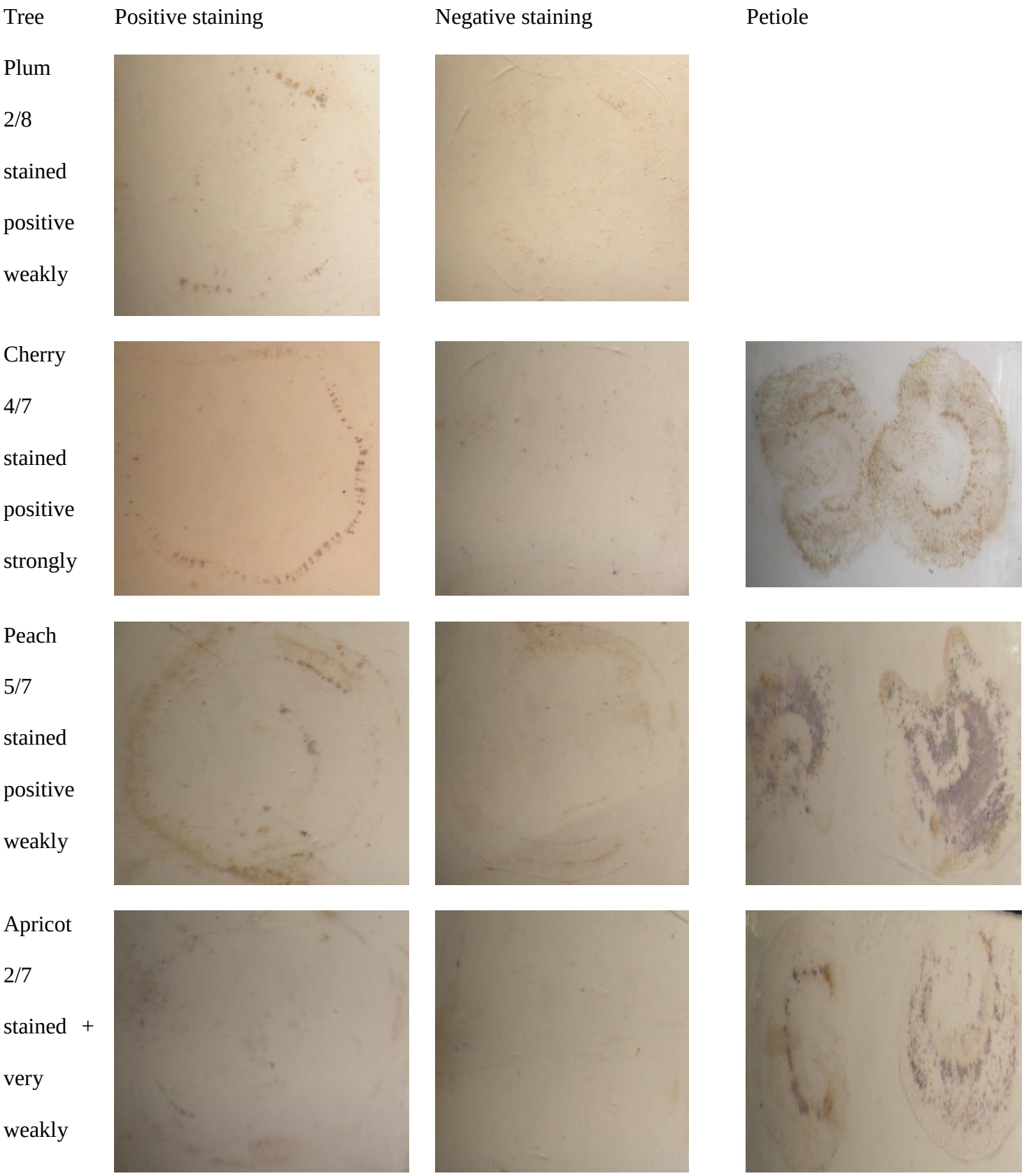
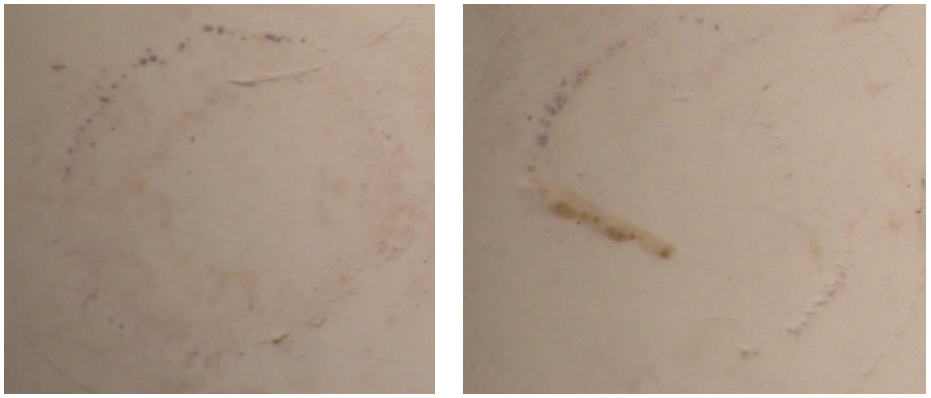


Figure 18: TBIA of stems and petioles of (A) multiple other stone fruits all sampled from the same irrigated mixed orchard in the Hammana area and (B) infected and healthy samples from different regions of Lebanon tested with anti-F8 antibody.

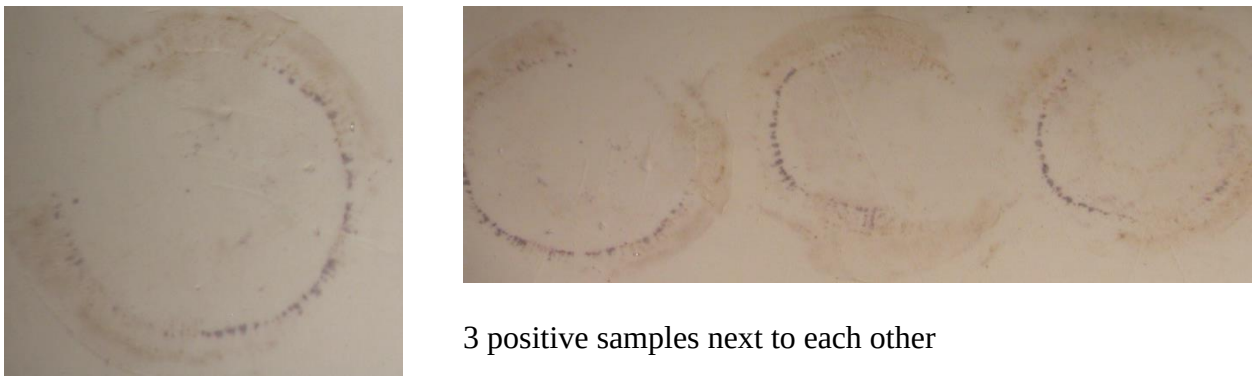
Almond 6/7 stained positive moderatly

B) Positive staining of different infected and healthy samples

Infected Almond thoum region 3/8 positive



Healthy Almond South Leb 7/8 positive



3 positive samples next to each other

All the trees in Figure 18.A were healthy with no visible symptoms and no AlmWB reported in the area. The infected and healthy trees in Figure 18.B were confirmed by nested PCR. The higher number and to an extent stronger staining of susceptible plants such as peach and almond in comparison to a weaker stain and less

Healthy Almond Bekaa region 8/8 positive

5 positive samples next to each other

number of stained samples in non-susceptible ones such as cherry and plum (Fig. 18, A) is very peculiar. However, it is not enough to jump to conclusions such as presence of phytoplasma or presence of a pathogenesis or transmission related protein. This is especially true because the symptomatic infected almond did not stain as strong as the healthy almond in Fig. 18.B but that might be, as said earlier, because it was from a weak non-irrigated tree at the end of the summer. To comment on the petioles in Figure 18.A, the color observed in healthy cherry petioles is not the NBT/BCIP purple stain and due to some cherry pigment. Petioles of healthy peach and apricot, however, have reacted extremely strongly with anti-F8 and lastly the same reaction can be seen to a lesser extent in the almond petioles from the same orchard.

To shed some light on which protein anti-F8 was reacting with, at least the size of the protein, we performed western blots with plant extracts from these infected tissues. Western Blotting (WB) with anti-F8 showed a faintly stained band along the dye front in the first trial. Therefore, in the second trial, a higher concentration of acrylamide was used in PAGE, but the anti-F8 did not react with any band; while, the F8 peptide used as positive control, stained strongly (Fig. 19). The low phytoplasma titer might also be the obstacle in WB. To improve the WB test, two options may be followed: either increasing the sensitivity of the serological test by employing a signal

amplifying system such as biotin-streptavidin or by using a method to concentrate the proteins in the plant extract, before gel electrophoresis.

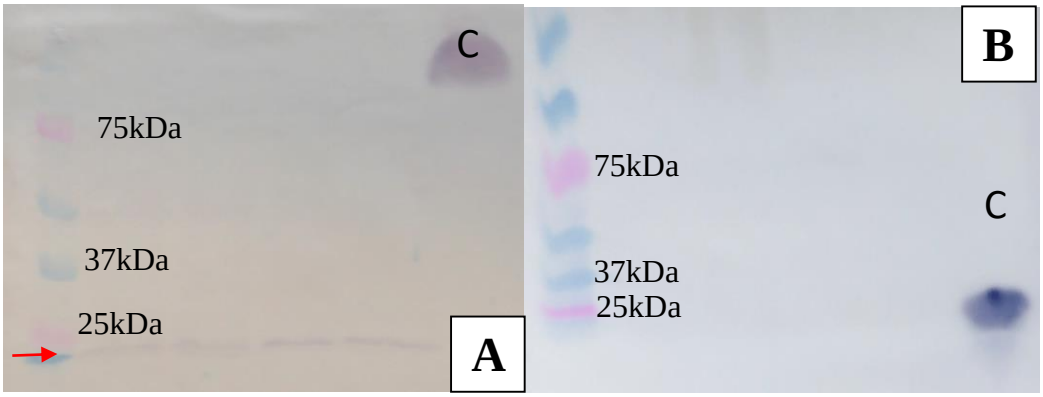


Figure 19: Western Blot of the TBIA positive samples with anti-F8 antibody. (A): Faint thin stained bands can be seen at the dye front of the gel indicated with a red arrow. The Control C peptide is high in this gel because it was loaded in the middle of electrophoresis. (B): A repeated run with no bands stained in the samples.

CHAPTER V
CONCLUSION AND RECOMMENDATIONS

AlmWB caused by '*Candidatus* Phytoplasma phoenicium' remains a devastating disease of stone fruits in Lebanon. The disease is very invasive capable of travelling undetected for long distances before the appearance of first symptoms. The EPPO has thus placed this organism under its A1 alert list with high phytosanitary risk for the endangered area. To quarantine a pathogen effectively, practical, sensitive, and specific

detection methods are a must. Detection of '*Ca.* P. phoenicium' currently relies on PCR or qPCR methods. These methods, while very sensitive and specific, are time and labor consuming as well as costly. Serological detection methods such as TBIA have proved ideal to overcome these drawbacks of nucleic acid-based detection methods in the diagnosis of many plant pathogens. TBIA simplicity and rapidity would be very valuable for early detection aiming at limiting the spread of AlmWB to endangered areas more efficiently and may be very useful for surveying the host range of the pathogen at a reasonable expense.

In this study, multiple '*Ca*. P. phoenicium' membrane proteins were identified as possible antigens for antibody production. Primers were designed for eight membrane proteins genes, or non-transmembrane truncated regions of three of these genes. These genes were amplified using PCR and cloned into two different expression vector/competent cell systems: pQE30 expression vector transformed into M15 competent cells, and pRSET A expression vector transformed into C43 competent cells specialized for the expression of toxic proteins such as membrane proteins. After sequence verification, protein expression was induced at a range of conditions with no detectable target protein expression observed after SDS-PAGE analysis, while thick bands corresponding to positive control protein were obtained, confirming the success of expression induction in the control. This led us to believe the membrane proteins were toxic to the *E. coli* cells or not suitable for expression in *E.coli*.

Thus, our focus shifted to attempting the expression of some of the membrane proteins in the PURExpress® 2.0 cell-free protein expression system. Reactions were set up as recommended by the kit manual and personal communications with the supplier. Two forms of DNA templates were tried: our target membrane protein genes cloned into

a T7 promoter expression plasmid (pRSET A) and/or, 6x-histidine tagged PCR products resulting from a 2-step PCR protocol which adds a RBS and T7 promoter site upstream of the target gene. Both plasmid and PCR product templates did not yield any detectable target protein in the reaction, while the control protein was well expressed. These results eliminate the possible cause of expression failure in the *E.coli* system to be cell toxicity or growth retardation. It can only be assumed that the phytoplasma gene sequences are not suitable to be transcribed/translated through *E. coli* cell machinery whether in vitro or in vivo. If any further tests are to be performed in *E. coli* based systems, codon optimization to suit *E. coli* expression is recommended.

As a last resort, three short (15-20aa) peptides were chosen from these membrane protein sequences based on their non-transmembrane location and predicted antigenicity. These sequences were sent to GenScript Biotech Corporation (Hong Kong/China) where they were synthesized. These peptides were conjugated to KLH and used as immunogens to raise polyclonal antibodies in rabbits. After affinity purification, the company sent us lyophilized antibodies that were tested against phytoplasma in serological assays such as TBIA, DBIA, and western blotting. Only one of the three antibodies reacted in a satisfactory manner in TBIA to a potential phloem antigen as revealed by NBT/BCIP staining. However, the same staining pattern was observed in healthy samples; this led us to believe that the antibody is not detecting a phytoplasma protein but most likely, a plant protein located in the phloem; without eliminating other possibilities. DBIA and western blots gave no positive results, suggesting that in the plant protein extracts used, the phytoplasma antigens are present at a very low titer and are diluted among a relatively large number of plant proteins making their detection very difficult. While in TBIA, the phytoplasma proteins are limited to the phloem tissue.

For future studies, the protein, with which the F8 anti-peptide antibody is reacting with, should be determined to understand the reason of the cross-reactivity in the healthy plants and subsequently help designing better antibodies against these pathogens. The best way to conduct these studies would be with high-sensitivity western blotting techniques. As for recombinant membrane protein expression, other promoters and expression systems such as in-planta expression systems or plant-based cell free systems can be tested. This goal remains important because these recombinant proteins will not serve only to raise antibodies but also to study their roles in pathogen-host-vector interactions. This is crucial to understand and better manage the pathogen.

Recently, LAMP field methods to detect plant pathogens including universal phytoplasma detection kits became commercialized and are gaining in importance. Therefore, developing a specific LAMP technique for AlmWB detection seems another attractive alternative, being much less time and material consuming than PCR and not requiring costly facilities and equipment. However, for fast screening purposes, because of automation and low cost, serological methods are still the superior option.

APPENDIX I

DNA SEQUENCES, PRIMERS, AND MAPS

A. DNA Sequences of chosen membrane proteins

1. F1 : AlmWB_01200 znuB ABC-type Mn/Zn transport system, permease (1068 bp)

ATGATCGATATAAATTCAATTTTTGGGTTATTAGCTACTTCTACTTC
TTTCAAAATTTGTTTAGGTGCTTTTTTACTAAGTATGACATCTGGGA
TTTTAGGTATTTTTATTAGCTTAAAAAATAAAGCTTTATTAGGTGAT
ATGCTTTCGCATGCAGTTTTACCAGGGATTGCCTGTTCCTATATTTG

GTTCCGAACTACTAACGAATGGGTAATTTGGTTAGGAGCTATTGGA
GCTTCTATTATATCTTTAAGTTTAATGGAATTAATTAAAAGATATTC
TAAAATCAAAACAGACACTATTCTTTCTTTAATTATGGCTTCTTTTT
TTGGTTTAGGTAATGTGTTAATTGCTTATGCACAAAAAGTTGCAAC
AGATAGTTCTATTGCTATTTTAGAAAAATTTCTCTTAGGCCAAATTG
CTTTAATTTCTGAAGAACATGTCAAAATCATAAGTATGATAACTCTT
TTAACTTTTTTAACCATTACTTTTTTATGGAAAGAATTTAAAATTTTT
ACTTTTGATGAGTTTTTTGCTAAAAGCATGGGATTCAATAATATCCT
AATATCTTTTATCTTAAATTCTTTATTAATAGGGTTAATTATAATTA
GTTTAAAAATTACAGGTATTATCGTGACTAGTGCTTTATTAATTATG
CCAGGAGTTATTGCGCGTTATTTAAGTGATAAATTATCCACTAATAT
CTTTATTGTGACTGTTATTGCTTTTTTATCAAGTTTTATTAGTATAAT
CATTAGCCTTCATATAGATAATATGCCCACAGGCCCTATCATTGTCA
TTATTAATACCATTTTCATTTTGTTAACTTATTTATTTGCGCCCAAAT
ATGGAATTTTAAAAAGATTTTTCAAACAAAAAAAATATAAACAACA
AATTAAAAAATTTCGACAATTAATACATTTTTACCATCATAATACAT
ATTATGAAATCCCTAAATTAGAAAGTTTTTTATTTAAAGAACAATA
TTTATATAAAACACCTCATCAAATTATCATCACTGCTAAAGGTATTC
AATTAGTAGAAAATTTAATAAATGGTAGAATTTAA

2. <u>F2</u> : AlmWB_02630 ugpE Glycerol-3-phosphate ABC transporter, permease 2 (912 bp)

ATGAAAGAAATTAACATTTCTTGTTTCTTTAAAAGAGTATGGAATA
GTATTAAGAAAAAGTATAAAAGTATTAGTTTTTTTGCGATATTAAA
ATATTCTTTTTTATTTTTTATTTCATGTTTTTTAACTTTAGCTTTTTAT
GCGATGTTTATTATGTCTTTGAAAAACAACGACGATATTATAAATA
ATAATATTTTATCGTTTCCTAAAAATGGTTGGCATTGGGAAAATTAT
CTATCAGCTTTCCAAGCCTTCAAATTTTTCCGATATCTTTGGAATAC
TTGTCTTATGGTATTTTTCTCGACTTTATTTGGAACTATAATATGTAT
TATTACAGCTTTTGCTTTAACCATGTTTGAATTTCCTTTAAAAAACG
TCGTTTTCAAGTTATTGTTATTAGGTTTAATGATTACTAGCGAAACC
TTAATTTTAACTAATTATCGTACAGTCGCTAATTGGGGGATGGTGA
ATGCTGGTTATGGCACTGAATTTCCTGGAGGAGTTTATTTTGCTATG
ACTTTACCTTATTTAATCAATATTGTTCATATCTTAATTTTAATAAG
AGCTTTTCAACGCGTTCCTAAAGAGTTATATTATACATCAAAAATT
GATGGTGCGACTGATTGGTATTATTTATGGAAAATTTTAGTACCCAT
TACAAAAGCTACTATTATAATTACTGTTATTTTTCGTATTGTTGCTG
CATGGAATGCTTATGCTTGGCCAGAATTAGTAGGTGGTGAATTATT
AACTAATATGGCTCGTAAAACGTTTAATAACGAATCAGGAATTGAC
GCTGTTAATATCCAAATGGCGATCGCTGTTTTGATTAATTTACCTTT
ATTTTTTATTTTTATATTCTTTAAAAAATATATTGTTTCTGGAGAAA
ATAGTAGTGGTATTAAAGGATAA

3. **F3 : AlmWB_02640 ugpA Glycerol-3-phosphate ABC transporter, permease 1 (930 bp)**

ATGTTTGATATTATAAATCAAAAAAATAATAAACATTGGTGGTATT
TATCTCCTGCTTTAATAATTTTGGTAATTTTTACTTTTTTTCCTTTAA
CGAAAACATTTATTATTTCTTTAAGTAGAGATTATAATAAATTTAAT
GATCATTTTACGGCTACATTTAATTTTGAAAATTACCGAAATGTTTT
TAAGGATCCAGAATTTCTTATATCTCTTCGCAATACTTTAATATTAG
TTTTTTTTACTGTTCCTATTTCTTTGTTTATCTCTTTAATAATCGCTTT
AACTTTGAATAGTATTCAAAACCGTTTTTTTAAGGATTTTTTAAAAA
CTTTTTTTTTCTTGCCATTATTATCTAATATAGTTATTATGGGTATGG
TATTTAGTATTATTTTTTATTATAACTATGAAATGGAAAATTATCCA
CAAGGTGTATTTAATAGTTTTTTATCTTCTGTTTTTCATATTAAACCA
CAACAATGGATAACTAATACAGCTCCTTACGAACATAAAATGTTTG
TATTGATTATTTATAATATTTGGACGCGTCTTCCTTTTAAAATTTTTG
TTTTTGTTCTTGCTTTACAAGATATTAATAAATCTTATTATGAAGCT
GCCAAAATAGATGGTGCTTCTCGTTTTCGTATTTTTTTTAAAATTAC
TTTACCATTACTGATTCCTATTATTTTTTATCAATTTATTATTGAAAT
GTTAGCAATTTTTAAAGAATACGAGTCAATTATAGGAATTTTTGCT
AATAATATTAATTATGAAATTAGAACTATTGTGGGTTATATTTATGC
TCAAACTTCTAATTTTTCTTATAATTCTTATTCAAAAGGAGCTACTG
CAGCAATGATTTTATTTTTTATTTCGGTTTTATTCACTGTTTTAAGTT
TTTATTTTTCGAAGAAAAAAATAAATTATTAG

4. **F4 : AlmWB_00730 hypothetical protein, putative cell surface protein (429 bp)**

ATGTGGTGGGGTCTTAGTTTAGTGAGTTTAGGTTATTATTGTTTTAA
TTTTTTAAAAGGTTATACCGTTAAAGAAATTTTAGAACGTCGTTTGG
ATACGATGCAAACAACGATTTTTACTGTTTTTTTAATTTTGGGGTTT
CAACCATTTTTTCTTTTTCTGCAAAAAGGGTTGATTTTCCTGAAAGA
TGTTTTTTTAGGTTGTTTTTTTCCTGAAAAAAAATTAAAAAATTTAG
AAATTAAATACGCCAAAGAGGTTTATCGCCATGAATTACTTTTAAA
AAAATTAGCTACTCTTCAAAACCAGCAACAAAAATTGCAACAAAA
AATTTTGACTCAACAACACAAAGTAGCTCAAAAGAAAAACCAGCA
AACTTTGAAAGACACTTTGGTCCAAAAAAAAGAATTGGAAGAGAA
AAAAAATGAGTAA

5. **F5 : AlmWB-1160 peptidase S24-like (564 bp)**

ATGAATCGAAACGTATGGCCAAAAATAAAAAAATTTTTTAATGTTC
TAAAGAATTGTCTTTTTATCTTTTTATATGCTTTATTATTTTATTTGA
CTTTAATCCAAATTAGCAATTTTATTAATCCTACAAGAACAGTGGA

TTATTTATTTTTTAATTTTTTTGAAGTTGCTAGCAGTAGTATGGAAC
CAGGTATAAAGAAAAGTGATAAAGTGATTTTAAAGAGAATTCACG
ATAGAAAAACATTGCAACCAGGTGATATTATTTATTTCGAAACCAG
TGATCCTTCATTGCAAAGTATCGGAATTAAACGTATTATTCATCGTG
TTGTTAAAAATGATAAACAAAACGAAGAAATTACAACTCATGGTG
ATAATAATGAAAAAATAGCGCCTTTTGAAAGACAAATACCTTATAA
GGATGTAATAGCCGAACATTTTTATACTATTCCTAACCAATATATCC
AAAAATTGAATATTATCTTTTGGTTTGGGTTTATTTATATGATCATT
GAACTTTTTTATAAAATTATTTATGTTAAAAATGATCCGAAAAATAT
TATTCTTTAA

6. <u>F6</u> : AlmWB-1850 hypothetical integral membrane protein (717 bp)

ATGACAGTTATGTTAAAATTAAATAAAGATAAACATTATTATTTCA
ACATTTGGCTGACTAAATGGTTTTGGATGATTTTTTATACATTACTT
TTATCTTTAGGAGTTTATTTTTTTACTTTTGGATTTCAATTAGTGACT
GGAGGGTTAGACGGTTTAACTGTCTTAACCATAGAAATTTTACAAA
ATTGTGGTCTGCCAAATGATTATATACCCCGAGTAGAATATTTATAT
GGATTTTACAATATTATTAGTTTAATAGCGGGATATAAGGTTTTTGG
TAAAGATTTTTGTTATCATACTGGTATTTTATGTATCATCTTATGTTT
AAGTGTTTCTTTTTTAAGTTGGCTTTTCGGCGACATTAGTATTGTAA
CCTGTTACCTCAGTCCTAATGATTATTTTAATTTAATTTTTGTTTCTA
TTGCAAGTGGTATCTTATTTGGTATTGCTTTAGGAAATATTCGTAAA
TATAAATATACCACTGGTGGAATGGATATTTTTCAAAAAATTTTAA
AAGATATTTATGGTATAAATTTTATTTGGGTTGTTTTCATAACTGAT
GGAGTTTTAATATTGCTAACAGCAATCATTATTGCTACGAAGAATC
ATGATTTAATGCAATTTATTATTAGATTTTTTTGCTCTTATTTATCTT
CTTTTATTATGAGTTGTATTATCGAAAAAATCGCTCCTGAAATTCAA
AGTGTTAAATAA

7. <u>F7</u> : AlmWB_00860 putative inhibitor of apoptosis-promoting Bax1 (BI-1)

(828 bp)

ATGAATTGGGAAAATAAACGTAATAAAAATACTTTTATTCGAACAA
ATCCTATTATAAATGATATTAAAAGAAATATAATACAAAAACGTGA
AACATATGCTTCTTCATCTGTTTTTAAAACTTGTGAATTTACAACTA
TAGGAATAAAAACTGTTTTTTTATTTTTATGTACCTTTGTGACTGCT
TTTCCTAGTGGTGTTTGTTTTTATGTTTTTCGTGACAACATGTATATA
CAAAGTTTCTTTATAACATTATTAATTGCATCTTTTTTAGTGCATATT
TTTTTATTATTTAAATTATCTTTCAATTCACACGCTATAGAAAAATA
CAAAAATTTAGTGATTATGTTTGCTTTGGTCCAAGGTATTAGTATTG
GCGCTAGTATATCTATCTTATATCAATCATTAGGTCCTACATACTAC
GATTTTGTTAACATGATATTATTAGCTTTTTTAATCACAGGTATCTT
ATTTATGGTTTCTCATTTTTTGTATTCTACTAATTTAATTAAAGTAAA
TCAAAAATTATATTTGTGTTTCATTGCTTCTTTTATTACTTTATTTTT
TATTATTTTTTTAAGTTGTTTCTTTTCATTTTTTAATAGTGTTGCCAT
AATGATTCCTATTACTTTATTTTGTTTATTTTTGGGTTGTATGATGAT

TTGTTTTGATTTACATAATGCAGAATTTATAGTTGAAAACAAGTTGC
CCAAAGAATATGAATGGAAAGTGGCTTTAGGTTTTCATATGACTTT
AATTTATATTTTTTTTCAAGCTATTCGTTTATTACAGTTATCAGGTAT
GTTTTCAAGACAAAATAATAGATAA

8. **<u>F8</u> : AlmWB_01190 sitD Manganese ABC transporter, inner membrane permease protein SitD (1101 bp)**

ATGTGGTATATAATAACAACTATTTTAAAGTCTTTTGAAACAGATA
TTTTTTGTATTTTAATTTTATGTTCTTTGTCTTTGGCCAATTTAGGTG
TCTTTTTAATTTTAAAAAAAGTGTCTATGGTGATAGATGCTATAAGT
CATAGTGTTTTATTAGGAATTGTACTAGCTTATTTAATAGTCAAAGA
TTTAAATTCTTCTTTTTTAATTATAGGAGCTACTTTAGTAGGTGTATT
AACTGTCTATTTAATAGAATTAATAAGTAAAAATAGTAAAATTTCC
AAAGACGCGGCTATTGGTATTATCTTTACTTTTTTCTTTTCCTTAGCT
ATCATTATTATTAGTATATTTATTAGAAATATCCATATAGATACAGA
TGCTGTTTTTTTAGGTAACATAGAATTAGCGCATAGTTCAAAATTAT
ATAAAATTATTCCTGTCTTAATATTAAATTTATTGTTTGTGATTATTT
TTTATAAAGAATTAAAGATTTTTGTTTTTGATCCTTCTTTAACAAAT
CTTTTAGGTTTTTCTTCCTTGTGGATTAACTATTCCTTAATGACTTTG
GTTTCTTTAACAACAGTTATTTCTTTTGATCTGGTTGGTTCCATCATG
ACTGTGGCTTGTATGATCGGCCCTGCAGCTACATCGCGACTTTTAAC
AAAAAGATTATTAACTTGTTGGCTTTTATCTTTATGGTTAGCTTTGG
TAAACACTAGTTTAGGTTATTATTTAGGAATTTATTTTGACCTAAAT
GTTTCTGGGATGATTGCTGTCGTTACTTTAGTAATTTTTTTAATTGTT
TTATTTTGTGAACCAAAAAAAGGTATTATATCAAAAACCATTACAA
ATTATTTTCATAAAAAAAATTTAATTTTAATTAATTTATTGATGCAT
TTAGAAAATAATTTAATACAAAAGAAAACAAATAATATTCAAAAT
ATTAAGAATGAATTAAAATGGTCTGAAGAAATTTTTCGAACATGTT
TGCGCAAAGCGCTGAAATCTAAATATATCATACAAAAAAATAAAA
AAATATTGATTACTTCTTTAGGTAAAAAATATTTATACCAAAAAAT
AAAATTAATTTTTAATCCTTAA

9. **<u>5N</u> : Truncated F5 (354 bp)**

AATCCTACAAGAACAGTGGATTATTTATTTTTTAATTTTTTTGAAGT
TGCTAGCAGTAGTATGGAACCAGGTATAAAGAAAAGTGATAAAGT
GATTTTAAAGAGAATTCACGATAGAAAAACATTGCAACCAGGTGAT
ATTATTTATTTCGAAACCAGTGATCCTTCATTGCAAAGTATCGGAAT
TAAACGTATTATTCATCGTGTTGTTAAAAATGATAAACAAAACGAA
GAAATTACAACTCATGGTGATAATAATGAAAAAATAGCGCCTTTTG
AAAGACAAATACCTTATAAGGATGTAATAGCCGAACATTTTTATAC
TATTCCTAACCAATATATCCAAAAATTGAAT

10. **<u>7N</u> : Truncated F7 (153bp)**

ATGAATTGGGAAAATAAACGTAATAAAAATACTTTTATTCGAACAAA
TCCTATTATAAATGATATTAAAAGAAATATAATACAAAAACGTGAAA
CATATGCTTCTTCATCTGTTTTTAAAACTTGTGAATTTACAACTATAG
GAATAAAAACT

11. <u>8N</u> : Truncated F8 (285 bp)

CCAAAAAAAGGTATTATATCAAAAACCATTACAAATTATTTTCATA
AAAAAAATTTAATTTTAATTAATTTATTGATGCATTTAGAAAATAAT
TTAATACAAAAGAAAACAAATAATATTCAAAATATTAAGAATGAA
TTAAAATGGTCTGAAGAAATTTTTCGAACATGTTTGCGCAAAGCGC
TGAAATCTAAATATATCATACAAAAAAATAAAAAAATATTGATTAC
TTCTTTAGGTAAAAAATATTTATACCAAAAAATAAAATTAATTTTTA
ATCCTTAA

12. <u>Inmp</u> : (621 bp)

TTGATAAAAATTAAAAATATCTCACATCATCCTCATTCATTTTTGAA
GCAAATTGTTATTATAGCTGTTTTATGTTCTTTATCTATTGTTTTATA
TGCCACTACTAATAAATTTTTTCTTTCTATACCTTTTACTATTCGTAA
ATTTGTGTTTTTAGATTTTGTGGGTATTATACCGTTTTTATTTATACC
TTTATATACTTCCAATAAAACTACCATTAAAGGTTTAGTTTTTCTAG
GAGTTAGTTTTTCGGAAGCTGTTGGTTATTTTATTTTTCGTTATAAA
GCATATCCTTTTTCATTTATTTTAAATTTTACTTATGGAATTTGTTGG
GGGTTGTTACCCAATTTTTTTTTCAAAAAAAATTATTCTTTTATAAA
AACTTATTTCTTGATTTTATTGTTATTTATTTTTCATTATTTATTCGTT
AATATTTTAGCGGCAGGCCTATATTTTCATTTTTTGACTGATACCAC
TGATAAGATTTCTATATTATCTTTTTTTATTTCTAATAAGAAATGTT
ATTATTTTGTTTTAAGGTTTTTTTCATTGCCTATAGTTTCTTTTGTTG
TCACTTTTTTATATTTTCGTATTAAATATCACATTATTATGTTATTTT
AA

13. <u>GroEl</u> : (1632 bp)

ATGGTTAAACAAATTCTTTTTGGTAAAGATGCGAGAAAAGAAATTT
TAAAAGGAGTTGATATTTTAAGTGATACTGTTAAATTAACTTTAGG
CCCTAAAGGAAATAATGTTATTTTGGAAACTAATAGTTATGAATCT
CCTTCTATTATTAATGATGGTGTTTCCATTGCCAAAGAAATTGAATT
ATCAAATCCTTATCAAAATATGGGTGCTAAATTAGTTTATGAAGCA
GCATCTAAAACTAATGATAATGCAGGCGACGGAACTACTACAGCTA
CTGTATTAGCACAAAAAATGATACATAAAGGGTTTCAATTTGTTAA
TTCTGGTGCTAAAGCTGTTTCAATTAAAGAAGGAATTATAAAAGCT
TCTCGAGAAGTAGTTACAAGACTTTTAGCTAAGTCAAAACCTATAG
AAACACAAGGTGATATCGAAAACGTAGCCACTATTTCTTCTGGTCA
AAAAGAAATAGGACATATTATAGCTTCAGCAATGGAGAAAGTGAC
TAAAAAAGGTGTTATTAGTGTAGATGAATCTAAAGGATTTGAAACT
GAACTAGAAGTAGTACAAGGTTTGAAATATGATAAAGGTTATATTT

CTCCTCATTTTGTCACCAACAAAGAAAATATGTCTGTAGATTTTGAA
CAAGCTGCAGTATTAGTGACAGATCATAAAATTAGTAATTTACAAG
AAATTCGTTCTCTTTTAGAAGAGGTAGTTAAAAATTCTACTCCTTTG
CTTATTATTGCTGCTTCTTTTGAAAATGATGCTATAGGTGCTTTGGT
TTTCAATAAAATTAGTGGAGTATTTAATGTAGTAGCTACTGAAGCT
CCTGGTTTTGGGGATAATCAAAGAGAGTTATTACAAGATATTGCTG
CTTTAACACAAGCTACTTTTATTTCTAAAGATTTGAATATGAAATTA
CAAAACGCTAACTCACAATATTTAGGTAAAATTAGTAAAGTGGTTA
TTAAAAAAGACGATACTGTATTAATAGGAACTGAAAAAAATAAAA
ACTTAGAAGAACGTATTCAAGAAATTGAAGCAAAAATACAAAAAG
CTCTAAATGAAAACGCAAGCGAATATGAGTTAAAAAATTTAAAATC
TCGATTAGCTAAATTATCCGGAGGTATTGCGATTATTAAAGTGGGA
GCCGCTACTGAAACTGAATTGAAAGAGAAAAAATTAAGAATTGAA
GATGCTTTAAATGCTACACAAGCAGCTATTACAGAAGGTATTGTAG
TCGGCGGCGGTAAAGCTTTAGTGGAAGTTTATAAAGAACTAAAAG
ATAATTTAATCGATTCAAACGTAGATATCCAAAAAGGTCTTAATAT
TGTTTTAGAAAGTTTATTAATACCTACTTATCAAATTGCAGAAAATG
CTGGTTTCGATGGTGATTCTGTGGTTAAAGAACAATTGAAACAAAA
AGATAACTATGGTTTTGATGCTAAAGAAGGACAATATGTTGATTTA
ATTAAAGAAGGTATTGTAGATCCTACCAAAGTCACAAGACAAGCTG
TTATCAATGCTTCTTCTATTGCAGCATCTATTATTACAGCTGGTGCT
GCCGTAGTGGCTGTGAAAGAAAAAAATGATGCATCTTTAACACCAA
ATCCACATAACGCTTTATAA

B. Primer pairs membrane proteins

Table 1: Locally designed Primers for cloning in pQE-32 and pRSET A expression vectors , and for Cell-Free Expression

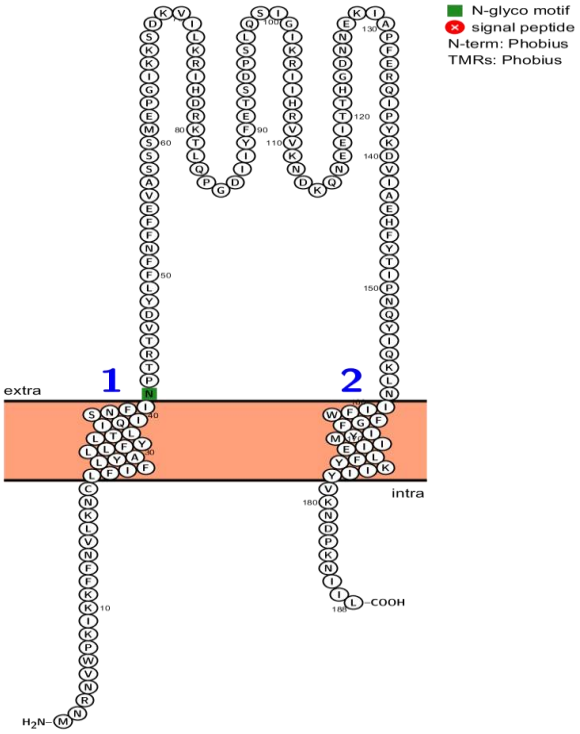
Primer	Sequence 5'-3' (Restriction Enzyme)	Gene/ Ann.Tm
Full protein expression in pQE-30		
F1	CGGGATCCATCGATATAAATTCAATTTTTGGGTTATT (BamHI)	F1 54.6ºC
R1	AACTGCAGTTAAATTCTACCATTTATTAAATTTTCTACTA (PstI)	
F2	CGGGATCCAAAGAAATTAACATTTCTTGTTTCTTTAAAAG (BamHI)	F2 44ºC
R2	AACTGCAGTTATCCTTTAATACCACTACTATTTTCT (PstI)	
F3	ACATGCATGCTTTGATATTATAAATCAAAAAAATAATAAAC (SphI)	F3 -
R3	AGGCCCGGGCTAATAATTTATTTTTTTCTTCGAAAAATAAAAA (SmaI)	
F4	CGGGATCCTGGTGGGGTCTTAGTTTAGTG (BamHI)	F4 57.4ºC
R4	AACTGCAGTTACTCATTTTTTTTCTCTTCCAATTC (PstI)	

F5	ACATGCATGCAATCGAAACGTATGGCCAAAAAT (SphI)	F5 57.3ºC
R5	AACTGCAGTTAAAGAATAATATTTTTCGGATCATTTTTAACATA (PstI)	
F6	ACATGCATGCACAGTTATGTTAAAATTAAATAAAGATAAAC (SphI)	F6 50.4ºC
R6	AATTCTGCAGTTATTTAACACTTTGAATTTCAGGAG (PstI)	
F7	ACATGCATGCAATTGGGAAAATAAACGTAATAAAAATACTTTT (SphI)	F7 60.5ºC
R7	AATTCTGCAGTTATCTATTATTTTGTCTTGAAAACATACCT (PstI)	
F8	CGGGATCCTGGTATATAATAACAACTATTTTAAAGTCTTTTGAAAC (BamHI)	F8 58ºC
R8	AATTCTGCAGTTAAGGATTAAAAATTAATTTTATTTTTTGGTATAAATATTTT (PstI)	
Truncated Protein Expression in pQE-30 or pRSET A		
5NF	CGGGATCCAATCCTACAAGAACAGTGGATTATT (BamHI)	5N 53.6ºC
5NR	AACTGCAGTTAATTCAATTTTTGGATATATTGGTTAGGA (PstI)	
7NF	CGGGATCCAATTGGGAAAATAAACGTAATAAAAATACTTTT (BamHI)	7N 56.4ºC
7NR	AATTCTGCAGTTAAGTTTTTATTCCTATAGTTGTAAATTCACAA (PstI)	
8NF	CGGGATCCCCAAAAAAAGGTATTATATCAAAAACC (BamHI)	8N 58ºC
8NR	AATTCTGCAGTTAAGGATTAAAAATTAATTTTATTTTTTGGTATAAATATTTT (PstI)	
Cell-Free Protein Expression with PCR Product		
CFBAXF	AAGGAGATATACCAATGCATCATCATCATCATCATGGTTCTCGTGAAACATATGCTTCTTC	CFBAX 57ºC
CFBAXF	GGATTAGTTATTCATTATTGTCTTGAAAACATACCTGA	
CFImpF	AAGGAGATATACCAATGCATCATCATCATCATCATGGTTCTATCTCACATCATCCTCATTC	CFImp 55.5ºC
CFImpR	GGATTAGTTATTCATTAAGTGACAACAAAAGAAACTATAG	
CFGroF	AAGGAGATATACCAATGCATCATCATCATCATCATGGTTCTCTTTTTGGTAAAGATGCGAG	CFGro
CFGroR	GGATTAGTTATTCATTATAAAGCGTTATGTGGATTTG	
T7Prom	GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCA	

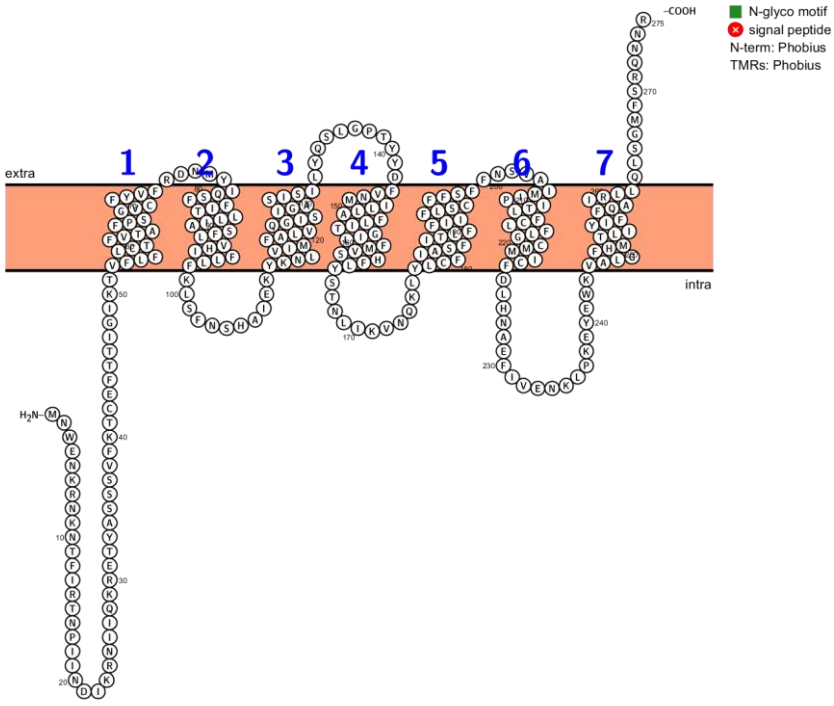
* The yellow highlighted regions correspond to the recognition sites of the restriction enzymes and the green highlighted regions are overhangs which facilitate Restriction enzyme activity.

C. Non-transmembrane Regions Determined By Protter

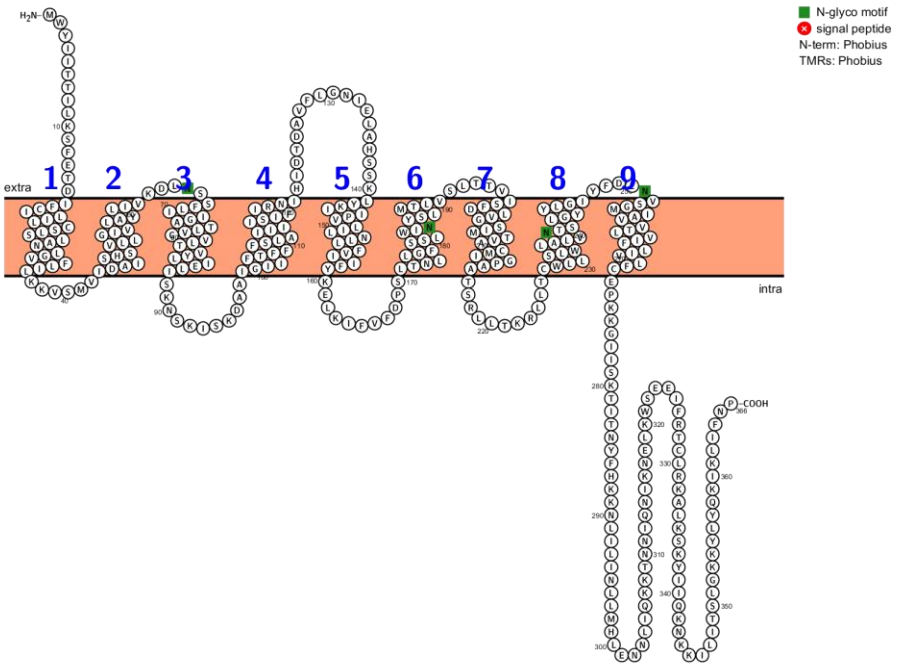
1. F5


N-glyco motif
signal peptide
N-term: Phobius
TMRs: Phobius
extra
intra
1
2
H2N-
-COOH

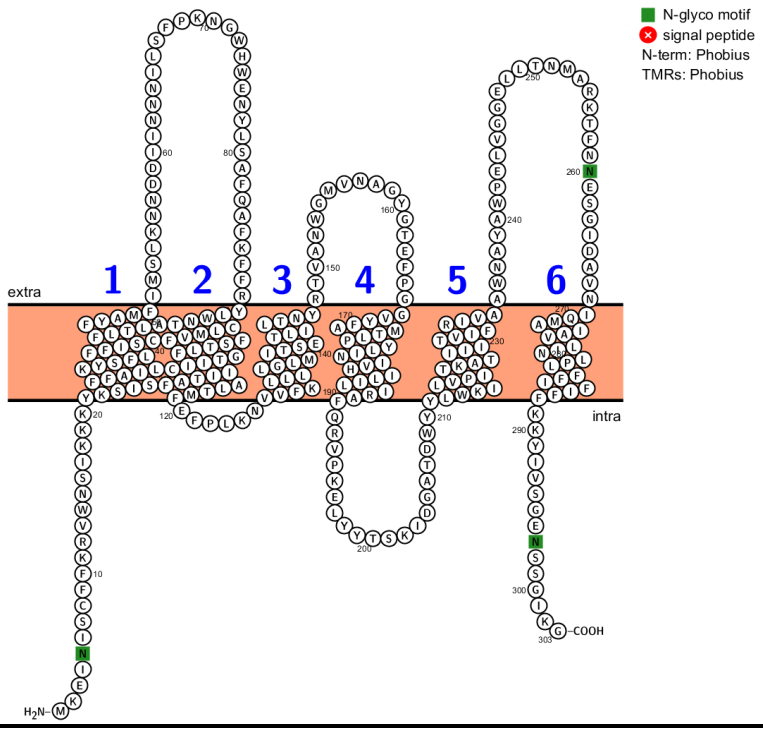
2. F7


N-glyco motif
signal peptide
N-term: Phobius
TMRs: Phobius
extra
intra
1
2
3
4
5
6
7
H2N-
-COOH

3. F8

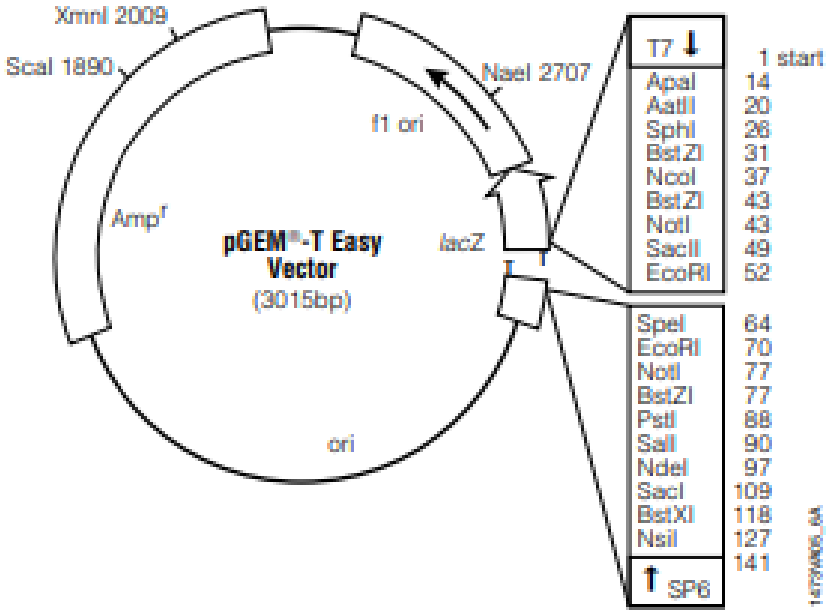


4. F2

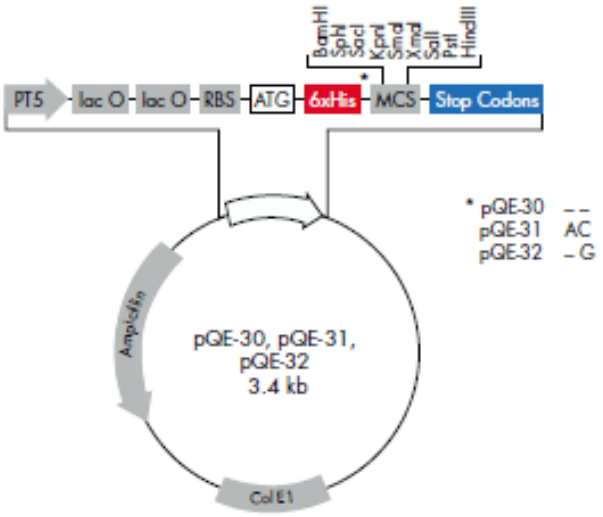


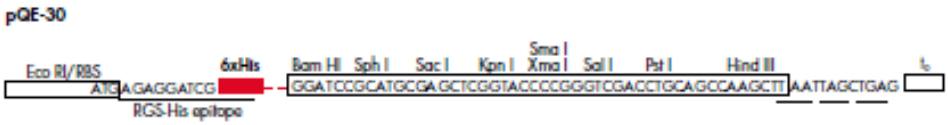
D. Vector Maps

1. pGEM®-T Easy (Promega) Cloning vector

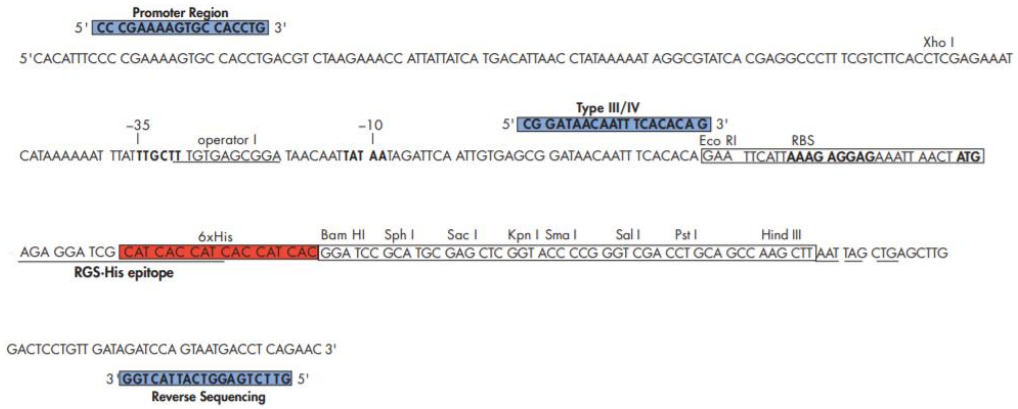


2. pQE-30 (Qiagen) Expression vector



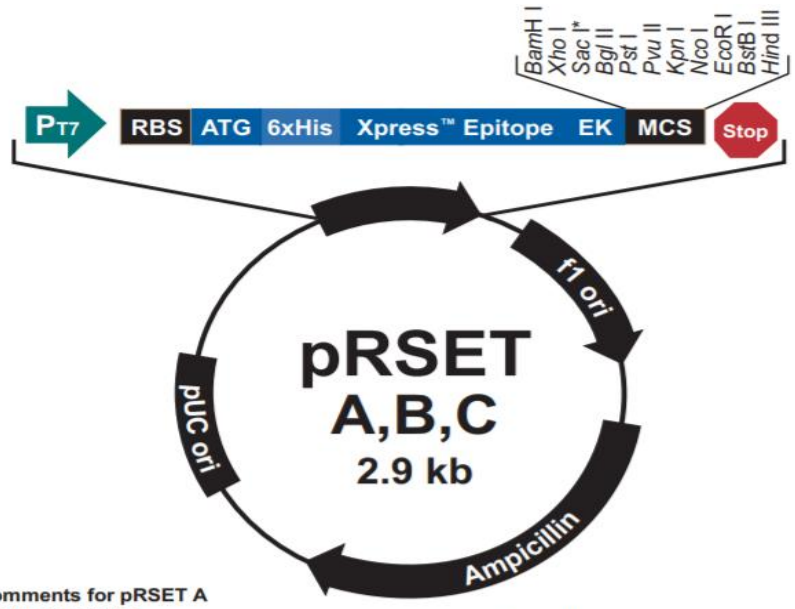


Sequencing primers for pQE vectors*



** Sequencing primers can be used for all pQE vectors except pQE-TriSystem.*

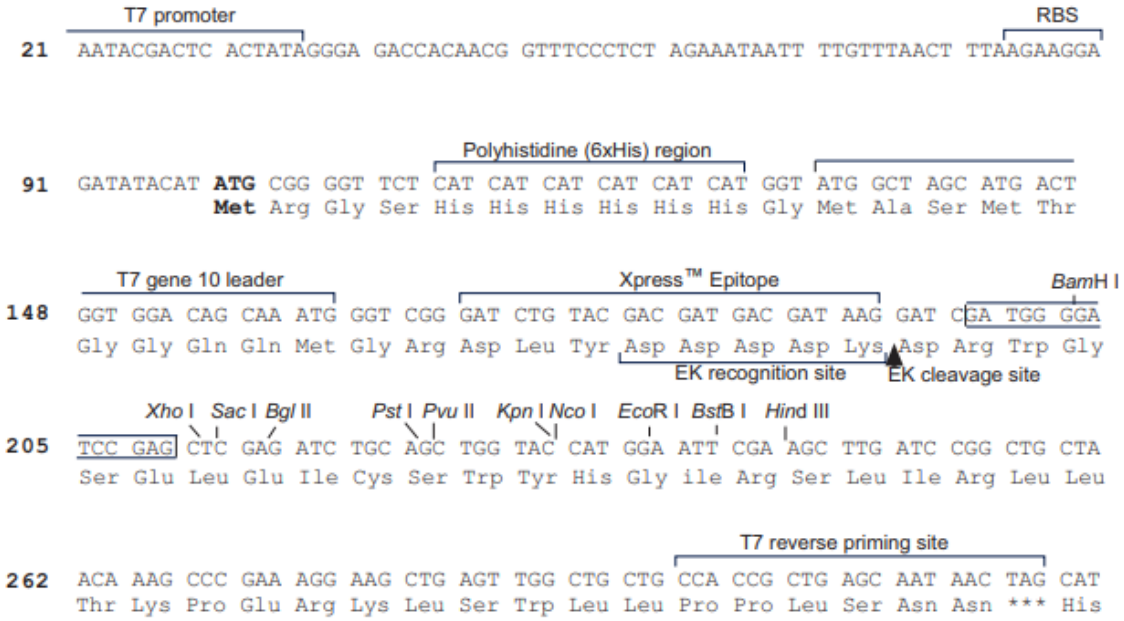
3. pRSET A (Invitrogen) Expression Vector



*Version C does not contain *Sac* I

Comments for pRSET A
2897 nucleotides

T7 promoter: bases 20-39
6xHis tag: bases 112-129
T7 gene 10 leader: bases 133-162
Xpress™ epitope: bases 169-192
Multiple cloning site: bases 202-248
T7 reverse priming site: bases 295-314
T7 transcription terminator: bases 256-385
f1 origin: bases 456-911
bla promoter: bases 943-1047
Ampicillin (*bla*) resistance gene (ORF): bases 1042-1902
pUC origin: bases 2047-2720 (C)



E. Peptide Sequences

F2: NAYAWPELVGGELLTC

F5: NEKIAPFERQIPYKC

F8: IHIDTDAVFLGNIELC

APPENDIX II

REAGENTS, MEDIA AND BUFFERS

A. 2% CTAB Buffer

2% w/v CTAB

100mM Tris

20mM EDTA

1% w/v PVP

1.4M NaCl

Dissolved in 100ml autoclaved distilled water .

pH adjusted to 8 with HCl.

B. X-Gal

200mg X-Gal in 20ml dimethylformamide. Stored at -20ºC.

C. M9 Minimal Media

90mM Na_2HPO_4

22mM KH_2PO_4

9mM NaCl

20mM NH_4Cl

In the case of agar and not broth add 1.5% w/v bacteriological Agar

Dissolved in 978ml of distilled water and autoclaved

Glucose (Final concentration 0.4% w/v) , $MgSO_4$ (Final 2mM), $CaCl_2$ (Final 0.1mM) and 5mg of Thiamine dissolved in 22ml solution were filter sterilized and added to the media after cooling to 50ºC.

D. Lysis Buffer

20 mM Na_2HPO_4

500 mM NaCl

pH 8.0

E. Urea Lysis Buffer

100 mM NaH_2PO_4

50 mM Tris

8 M Urea

pH 8.0

F. Guanidine hydrochloride Lysis Buffer

100 mM NaH_2PO_4

50 mM Tris

6 M Guanidine hydrochloride

0.5% Triton X-100

pH 8.0

G. 2xSDS loading buffer

2ml 0.5M Tris HCL pH 6.8 (100mM)

2ml 20% SDS (4%)

0.02g bromophenol blue (0.2mM)

2ml glycerol (20%)

2ml distilled water

500µl 1M DTT

Store in -20ºC

H. <u>12% SDS-PAGE Gel</u>

1. Separating gel

1.6ml 40 % Acrylamide

1ml 1.5M Tris pH 8.8

1.4ml DDiH2O

50µl 10 % SDS

70µl 10% APS

10µl Temed

2. Stacking gel

375µl 40 % Acrylamide

625µl 0.5M Tris pH 6.8

1.75ml DDiH2O

22.5µl 10 % SDS

70µl 10% APS

10µl Temed

I. <u>Running Buffer (1L)</u>

6.04g Tris base

37.6g glycine electrophoresis grade

20ml 10% SDS

Adjust pH to 8.3

J. <u>Coomassie Blue Staining Solution(500ml)</u>

225ml millique water

225ml methanol

50ml acetic acid

1.25g Coomassie Brilliant Blue

K. <u>Destaining Solution(500ml)</u>

225 ml millique water

225 ml methanol

50 ml acetic acid (fixes color)

L. <u>Tris Buffer Saline (TBS)</u>

20mM Tris

0.5M NaCl

Adjust pH to 7.4

For TBST add 0.05% Tween 20

M. <u>Phosphate Buffer Saline(PBS)</u>

800mg NaCl

$Na_2HPO_4.12H_2O$

KH_2PO_4

KCl

2% PVP

0.1% Na2SO3

For PBST add 0.05% Tween 20

N. <u>Western Blot Extraction Buffer</u>

100mM Tris

4% SDS

4% 2-mercaptoethanol

pH 6.8

O. Western Blot Transfer Buffer

25mM Tris

192mM Glycine

20% methanol

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