# PHAGE DISPLAY SCREENING AND EXPRESSION IN PLANTS OF PEPTIDE APTAMERS THAT BIND TO PTHA

By

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TO MY PARENTS

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The bacterial genus *Xanthomonas* is comprised of plant-associated bacteria, many of which cause severe plant diseases. One of those is citrus canker disease, caused by *Xanthomonas citri*. *X. citri* causes citrus canker by injecting a protein signal molecule, PthA, into citrus cells, and it is thought that several other severe *Xanthomonas* diseases are similarly caused by other members of the *avrBs3/pthA* gene family. This work was to determine if peptide aptamers could be selected that bind to PthA. A Phage Display Peptide Library was screened to select 7-peptide aptamers that specifically bind to full-length PthA and also to a truncated PthA consisting of the C-terminal 200 aa. Three

different aptamer sequences were identified that bound to both full-length PthA and truncated PthA protein. ELISA tests indicated that there were no significant differences in the binding affinities of the three aptamers. Based on the citrus codon usage preference table, four PCR primers were designed and used to clone the aptamer sequences into the plant expression vector pBI221. The aptamers were genetically engineered as leader sequences fused to GUS and driven by a CaMV 35S promoter. The engineered constructs were transferred into *Agrobacterium tumefaciens* strain GV2260 and inoculated onto California light-red kidney bean and sweet orange leaves. The results showed that one aptamer, of sequence YPASYMQ, had a strong effect to block the Hypersensitive Response (HR) normally elicited by *pthA* expressed in beans, and the pathogenic response normally elicited by *pthA* on citrus. Another aptamer, of sequence HPYTFLN, had reduced, but significant similar effects. Neither aptamer affected the HR symptom elicited by expression of *pthA* in tomato cells. This result is in consistent with other published reports that the COOH-terminal region of other members of the *pthA* gene family are not important for elicitation of the HR in tomato. These results confirmed that the aptamers exerted their effect by binding to the COOH-terminal end of *pthA*, and indicated that aptamers might be used to control citrus canker disease and several other severe diseases caused by *Xanthomonas*.

# CHAPTER 1
# INTRODUCTION

## Citrus Canker History

According to the Florida Citrus Outlook 1998-99 Season Report (The Economic and Market Research Department, Florida Department of Citrus, 1998), total Florida citrus production, including round orange, specialty-citrus and grape fruit is expected to be 248.8 million 90-lb boxes. The total 1998-1999 earnings are projected to be $971.5 million to $1,221.2 million. The Florida citrus industry is the largest among all US states - about 3 times more than the total citrus production in California, the second largest citrus provider in the US.

This important industry to the state of Florida is now threatened by citrus canker - a world-wide citrus disease, widely distributed in Southeast Asia, Japan, the Middle East, Africa and South America (Swarup *et al.*, 1991). Last year, Asiatic citrus canker infected large areas of southern Florida, including Hendry, Dade and Broward counties. About $200 million this year was set aside for canker eradication in Florida.

A tree infected by citrus canker will drop fruit prematurely and exhibit lesions on leaves, stems and fruit (De Feyter *et al.*, 1993) (Figure 1). The bacteria responsible for the canker, *Xanthomonas citri* (Gabriel *et al.*, 1989), is spread primarily by wind-blown

rain, but also can be spread by contaminated tools and equipment, by people and by animals. There is no effective resistance in grapefruit and little resistance in sweet orange. Control by chemical sprays is only partially effective. Control by eradication has been effective and *X. citri* is a quarantined pathogen, subject to eradication. Both infected and exposed trees are destroyed as part of a typical eradication program. If citrus canker cannot be contained in Florida, losses of $8.55 billion in revenue is expected according to the Florida Citrus Outlook 1998-99 Season Report. As many as the 121,000 people could be unemployed, and 845,260 acres of citrus trees could be destroyed. In 1985, an earlier outbreak of citrus canker in Florida forced the destruction of 20 million citrus trees.

Figure 1. Citrus canker symptoms on sweet orange.

Due to this situation, researchers at USDA, the University of Florida, are working to find a way to stop citrus canker. The primary purpose of this research was to try to find a molecular biological approach to cure citrus canker disease and a possible way to genetically engineer “immune” or “resistant” citrus trees.

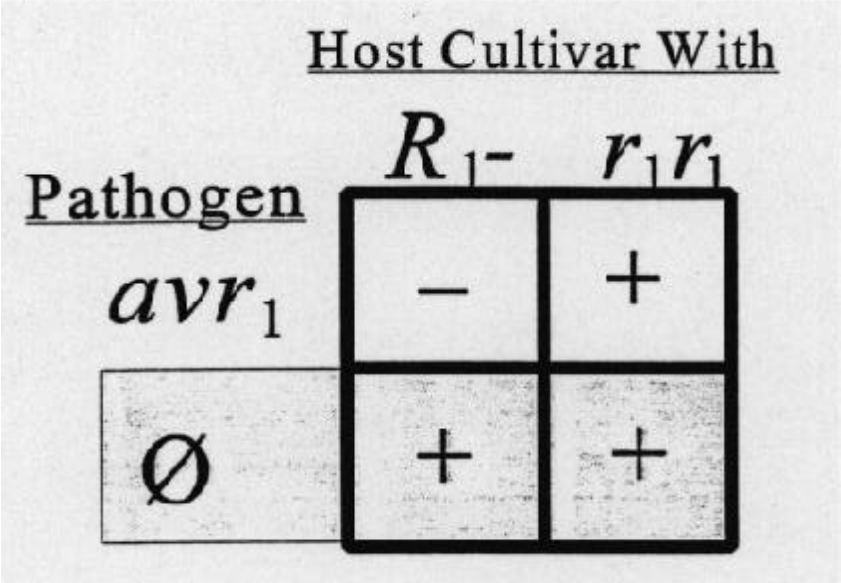



Figure 2. The gene-for-gene “quadratic check”.

## *Xanthomonas avr/pth* Family

The genus *Xanthomonas* is unique among bacterial plant pathogens in exhibiting a high degree of host-range specificity. The basis for the specifications is not clear. Based on the classic “The gene-for-gene quadratic check” (Figure 2) (Ellingboe, 1976), avirulence genes *(avr)* are considered to contribute to host range specificity in *Xanthomonas* (Gabriel, 1997). Resistance in plants or avirulence in pathogens is usually

assayed by inoculation of pathogen into plants and is usually seen as a hypersensitive response (HR) of the plant (Alfano and Collmer, 1996, Leach and White, 1996). Surprisingly, most of the *X. campestris* pv. *malvacearum avr* genes are also pathogenicity (*pth*) genes (Yang *et al.*, 1996). Even more surprisingly, these genes are members of the same *avr/pth* gene family as the genes of *X. citri* that are known to cause citrus canker (Swarup *et al.*, 1992, Duan *et al.*, 1999). Most of the members of this *avr/pth* gene family were originally isolated as *avr* genes and there was no evidence of *pth* functions. Similarly, *pth* genes were cloned by screening of pathogenecity, without evidence of *avr* functions. Currently, 17 members of this gene family has been published, including *X. campestris* pv. *malvacearum* (cotton blight) (Yang *et al.*, 1996), *X. citri* (Asiatic citrus canker) (Swarup *et al.*, 1991), *X. campestris* pv. *aurantifolii* (false citrus canker and Mexican lime cancarosis) and *X. oryzae* (rice blight) (Leach *et al.*, 1996). The genes cloned include *avrBn* (Gabriel *et al.*, 1986), *avrb6* (water soaking on cotton), *avrB4, avrb7* (water soaking on cotton), *avrBIn* (water soaking on cotton), *avrB101* (water soaking on cotton), *avrB102* (water soaking on cotton) (De Feyter *et al.*, 1993), *avrB103, avrB104* (water soaking on cotton), *avrB5* (water soaking on cotton) (Yang *et al.*, 1996), *pthN* (water soaking on cotton), $pthN_2$ (water soaking on cotton) (Chakrabarty *et al.*, 1997), *avrBs3* (Bonas *et al.*, 1989), *pthA* (canker on citrus) (Swarup *et al.*, 1992), *pthB* (canker on citrus), *pthC* (canker on citrus) (Yuan and Gabriel, unpublished data), *avrXa5, avrXa7* (elongated lesion on rice) and *avrXa10* (Hopkins *et al.*, 1992).

The *avr/pth* gene encode signals affecting plant cell programs (Gabriel, 1999), including “programmed death” (Dangl *et al.*, 1996). All genes of the *avr/pth* family

found until today are from biotrophs which produce few degrading enzymes that could assist releasing nutrient from living plant cells but not kill them (Vivian and Gibbon, 1997). A type III protein secretion system encoded by *hrp* genes (the name came from affecting both hypersensitive response and pathogenicity of the bacterial strains) is essential to deliver the *avr/pth* signals into plant cells (Van Gijsegem *et al.*, 1995). Mutation of this system abolish pathogenecity in biotrophic plant pathogenic *Erwinia*, *Pseudomonas* and *Xanthomonas* (Alfano and Collmer, 1996). All of these bacterial pathogens have very similar *hrp* genes (Galan, 1996).

Only a few molecules have been determined to be secreted by the *hrp* system. One of these is a class of toxin-like molecules called harpins (Hoyos *et al.*, 1996). Harpins directly elicit plant symptoms on both host and nonhost plants. Harpins are glycine rich proteins that seems to cause direct nutrient leakage from plant cells by alkalization of the apoplast.

Another group of secreted molecules are the *avr/pth* gene family (Mecsas and Strauss, 1996). Functional nuclear localization signals (NLSs) were found in all members of *Xanthomonas avr/pth* gene family (Yang and Gabriel, 1995). This is evidence that the signaling protein is directly targeted to plant cell nuclei. Site-directed mutagenesis of NLS sequences abolished the pathogenicity phenotypes expressed by these genes (Gabriel *et al.*, 1996).

## PthA Protein Function

Citrus canker disease has been historically described as having different “forms”, including Asiatic citrus canker (*X. citri* or “A form”), false citrus canker (*X. campestris* pv. aurantifolii form B or “B form”) and Mexican lime cancrosis (*X. campestris* pv. aurantifolii form C or “C form”). A pathogenicity gene, *pthA*, was first cloned by on pZit45 and confirmed necessary to for *Xanthomonas citri* to cause citrus canker disease, the hyperplasia of citrus tissue (Swarup *et al.*, 1991). The full length peptide sequence of

```
   1 mdpirsrtps parellpgpq pdgvqptadr gvsppaggpl dglparrtms rtrlpsppap
  61 spafsagsfs dllrqfdpsl fntslfdslp pfgahhteaa tgewdevqsg lraadapppt
 121 mrvavtaarp prakpaprrr aaqpsdaspa aqvdlrtlgy sqqqqekikp kvrstvaqhh
 181 ealvghgfth ahivalsqhp aalgtvavky qdmiaalpea theaivgvgk qwsgaralea
 241 lltvagelrg pplqldtgql lkiakrggvt aveavhawrn altgaplnlt peqvvaiasn
 301 iggkqaletv qrllpvlcqa hgltpeqvva iasnggkqal etvqrllpvl cqahgltpeq
 361 vvaiasnigg kqaletvqrl lpvlcqahgl tpeqvvaias niggkqalet vqrllpvlcq
 421 ahgltpaqvv aiasniggkq aletvqrllp vlcqahgltp eqvvaiasng ggkqaletvq
 481 rllpvlcqah gltpdqvvai ashdggkqal etvqrllpvl cqahgltpqq vvaiasnggg
 541 kqaletvqrl lpvlcqahgl tpeqvvaias hdggkqalet vqrllpvlcq ahgltpeqvv
 601 aiasngggkq aletvqrllp vlcqahgltp eqvvaiasng ggkqaletvq rllpvlcqah
 661 gltpeqvvai asngggkqal etvqrllpvl cqahgltldq vvaiasnggg kqaletvqrl
 721 lpvlcqahgl tpeqvvaias nsggkqalet vqrllpvlcq ahgltpdqvv aiashdggkq
 781 aletvqrllp vlcqahgltp eqvvaiashd ggkqaletvq rllpvlcqah gltpeqvvai
 841 acngggkqal etvqrllpvl cqahgltpeq vvaiasnggg rpalesivaq lsrpdpalaa
 901 ltndhlvala clggrpalda vkkglphapa likrtnrrip ertshrvadh aqvvrvlgff
 961 qc***hshpaqaf ddamtqfgms rhgllqlfrr vgvtelears gtlppasqrw drilqasgmk***
1021 ***rakpsptstq tpdqaslhaf adslerdlda pspthegdqr rassrkrsrs dravtgpsaq***
1081 ***qsfevrapeq rdalhlplsw rvkrprtsig gglpdpgtpt aadlaasstv mreqdedpfa***
1141 ***gaaddfpafn eeelawlmel lpq***
```

Figure 3. PthA full length peptide sequence (1163 amino acids in total, the carboxyl terminal 200 aa truncated protein is indicated by bold-italic).

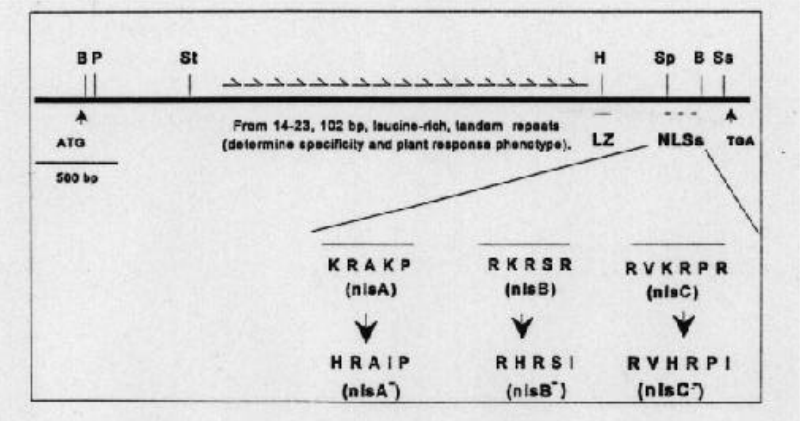



Figure 4. The predicted functional domains of PthA.

PthA is given in Figure 3 (Swarup *et al.,* 1992). There are 1163 amino acids. *pthA, avrb6* and *avrBs3* are members of *Xathomonas avr/pth* family (Yang *et al.*, 1994). The complete DNA sequence of *pthA* is 97% identical to *avrb6* and *avrBs3.* The central regions of the predicted PthA protein is comprised of 17.5 tandem repeats each 34 amino acids in length (Figure 4). These repeats are leucine-rich, indicating a potential protein-protein interaction. Also, leucine zipper-like heptad repeats are present, that may also indicate protein-protein or protein-DNA interactions. Site-directed mutagenesis of such repeats proved that they are critical for the function of cultivar specific avirulence and species-specific pathogenicity (Yang *et al.*, 1994). The repeat regions of *pthA* and *avrb6* determined hyperplasia of citrus and water soaking of cotton, respectively. Fusion experiments using *pthA* and *avrb6* repeat regions showed that the direct tandem repeats determine both phenotypes, and are host-specific. The coding sequences of all members of the gene family are flanked by nearly identical 62bp terminal inverted repeats, and the

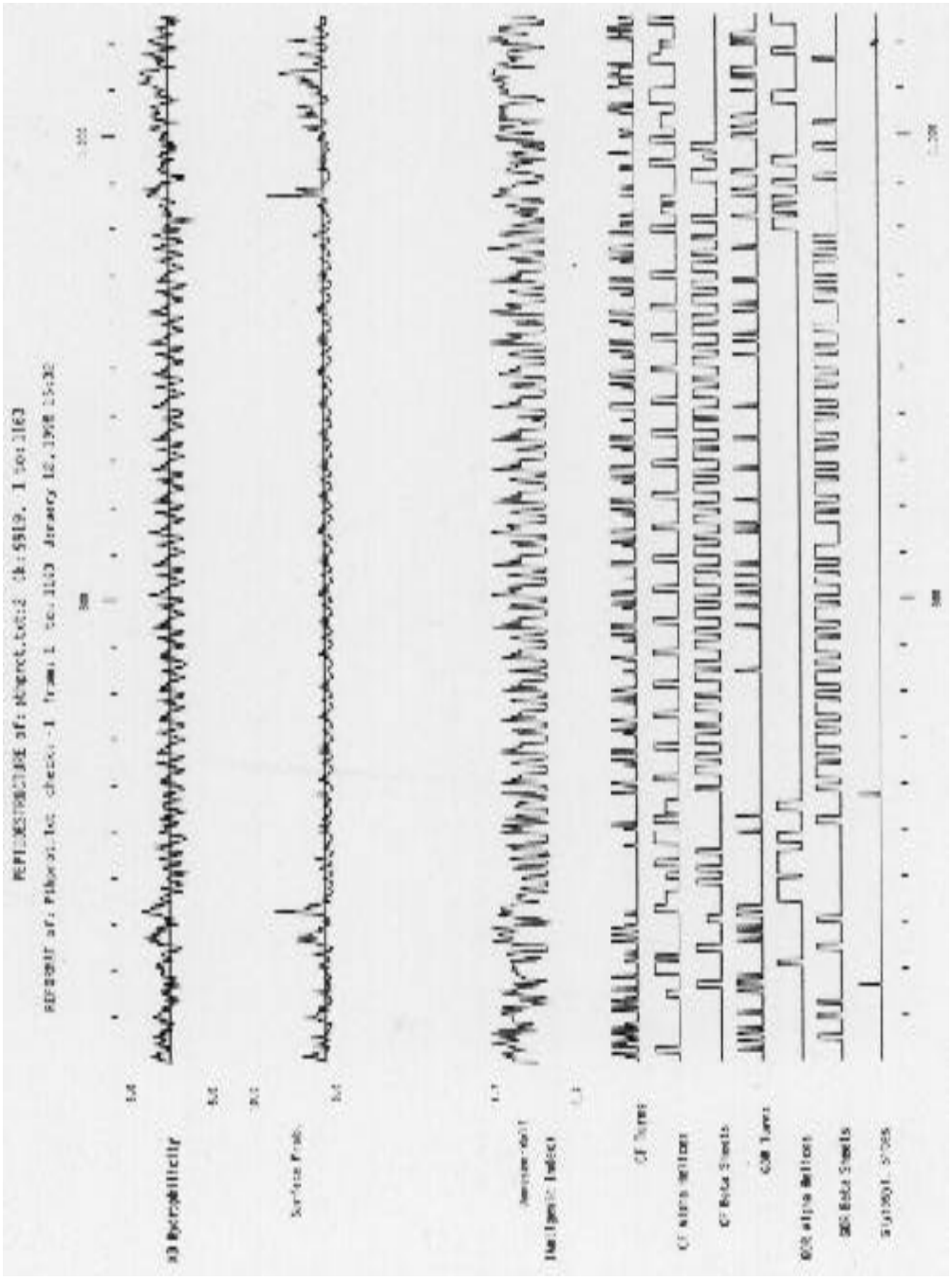


Figure 5. The predicted peptide structure of PthA (including KD Hydrophilicity, Surface Probability, Antigenic Index, CF Turns, Alpha Helices, Beta Sheets and Glycosyl Sites).

terminal 38bp of both inverted repeats are highly similar to the 38bp consensus terminal sequence of the Tn3 family of transposons (De Feyter *et al.*, 1993). Therefore, these genes may have the capability to transpose.

The carboxyl terminal portion of PthA encodes three nuclear localization signals (K-R/K-X-R/K) that are critical for PthA function and for localization to the host cell nucleus (Yang and Gabriel, 1995). These three putative nuclear localization sequences are at positions 1020-1024 (K-R-A-K-P) (nlsA), 1065-1069 (R-K-R-S-R) (nlsB), and 1101-1106 (R-V-K-R-P-R) (nlsC) in PthA. Site-directed mutagenesis of the nuclear localization signal reduced the *pthA* localized in onion cell nuclei and also reduced the pathogenicity of citrus canker symptoms (Gabriel *et al.*, 1996). Mutation of all three NLSs reduced *pthA* localization to onion cell nuclei. Mutations of any two NLSs abolished the ability the *pthA* to elicit cankers on citrus, but did not abolish its ability to elicit a non-host HR on tomato. Another member of the gene family from *X. campestris* pv. *vesicatoria, avrBs3-2,* does not require the 3' end (encoding all three NLSs) to elicit a HR on tomato. Because *avrBs3-2* and *pthA* are in the same gene family, and the DNA sequence are very similar, it is predicted that PthA C-terminal region is also not important to elicit a HR on tomato (Gabriel et al., 1996). The DNA coding sequence for the C-terminal regions of *pthA* was fused to a β-glucuronidase (GUS) reporter gene. Figure 5 shows the predicted structure of PthA full-length protein including KD hydrophilicity, surface probablity, antigen index, CF turns, alpha helics, beta sheets, and glycosyl sites, *etc.* Both the N-terminus and Carboxyl terminus of PthA are likely to be

exposed outside of the naturally folded protein, thus, the COOH-terminus of PthA may have the chance to interact with other protein or DNA molecules.

## Fusion Protein Construction, the pET-19b Expression System and the GST Gene Fusion System

The pET system used in the expression of recombinant proteins in *E. coli* was originally constructed by Studier and colleagues (Studier and Moffatt, 1986, Studier *et al.*, 1990). The pET19b vector provides a translational N-terminal fusion of a cloned gene to a cleavable His.Tag sequence for rapid affinity purification of the protein product. His.Tag is an oligohistidine domain that allows convenient, economical purification by His.Bind affinity resin. The target gene is cloned in the pET plasmids under the control of a T7 promoter which is induced by T7 RNA polymerase in the host cells (Dubendorff and Studier, 1991) . T7 RNA polymerase is highly selective, which converts almost all of the cell's protein production to target gene expression (Derman *et al.*, 1993). The expected yield of target protein can be more than 50% of the total protein production after induction. Target genes are initially cloned in *E. coli* hosts that do not contain the T7 RNA polymerase gene, *i.e.*, the target genes are silent in the non-expression host. When these plasmids are transferred into the expression host, *E. coli* BL21(DE3)pLysS, which contains a chromosomal copy of the T7 RNA polymerase gene, under *lac*UV5 control, expression of the target gene is strongly induced by the addition of IPTG (Doherty *et al.*, 1995). Since some proteins naturally occurred in *E. coli* host cell

having Histidine rich sequences, for some His.Tag constructs, they are not quiet efficient to bind to His.Bind affinity resin or have very high background which create the difficulties for the further purification of His.Tag proteins. Also, the very short peptides are not very efficiently purified by His.Tag approaches (Studier *et al*., 1990).

The *Schistosoma japonicum* Glutathione S-transferase (GST) gene encodes a 26kDa protein that is expressed in *E. coli* with full enzymatic activity (Parker *et al*., 1990). Recombinant genes are constructed in pGEX vectors, designed for inducible, high-level intracellular expression of genes in fusion with GST (Maru *et al.*, 1996). GST fusion proteins have GST enzymatic activity (Ji *et al.*, 1992). Gene expression is under the control of the *tac* promoter, which is induced using the lactose analog IPTG (Kaelin *et al.*, 1992). After lysis of bacterial cells, the lysates are incubated with Glutathione Sepharose 4B affinity resin. The glutathione group of GST is attached to Sepharose 4B by coupling to the oxirane group using epoxy-activation. The GST fusion protein can be 90% recovered using Glutathione Sepharose 4B in a single chromatographic step. Fusion proteins are recovered from the matrix under mild elution conditions (10mM glutathione) that preserve antigenicity and functionality of the proteins. Specific protease cleavage sites allow separation of the GST and the fusion protein to yield separate products. The GST fusion protein system proved to be very efficient for short peptide expression (McTigue *et al*., 1995).

## Phage Display Peptide Library - Biopanning

There are several rapid and efficient methods using combinatorial biology for screening random peptide epitopes binding to specific protein targets (Scott and Smith, 1990). The Ph.D.-7 Phage Display Peptide Library (New England BioLabs) is based on a library of random 7-mer peptides fused to a minor coat protein (pIII) of the filamentous coliphage M13. The Ph.D-7 peptide library is comprised of $2 \times 10^9$ sequences. By comparison, theoretical numbers of possible 7-mers in a combination of 20 different amino acids is $20^7 = 1.28 \times 10^9$ possible 7-residue sequences. Each amplification cycle yields about 100 copies of each amplified sequence in 10µl of the supplied phage. The displayed hetapeptides are expressed directly at the N-terminus of the pIII coat protein when the fusion is expressed with a leader sequence removed during secretion, therefore, the first residue of the mature protein is the first randomized position (Figure 6). The peptide is followed by a short spacer (Gly-Gly-Gly-Ser) and then the wild type pIII sequence. The genetic code of the Ph.D. phage display library is reduced, using only 32 codons encoding 20 different amino acids (Table 1).

Biopanning is a *in vitro* selection process that allows rapid identification of peptide ligands for a variety of target molecules (such as antibodies, enzymes, cell-surface receptors, *etc*) (Schumacher *et al.*, 1996, Goodson *et al.*, 1994, Barry *et al.*, 1996, Parmley and Smith, 1988). Biopanning is carried out by incubating a large random library of unselected phage-displayed peptides with a polystyrene plate coated with the target. After washing away the unbound phage, some peptides stick to the target and

Table 1. The genetic code for phage display peptide library biopanning.

| | 2nd T | 2nd C | 2nd A | 2nd G | |
|---|---|---|---|---|---|
| 1st T | Phe (F)<br>Leu (L) | Ser (S)<br>Ser (S) | Tyr (Y)<br>Gln (Q) | Cys (C)<br>Trp (W) | 3rd T<br>3rd G |
| 1st C | Leu (L)<br>Leu (L) | Pro (P)<br>Pro (P) | His (H)<br>Gln (Q) | Arg (R)<br>Arg (R) | 3rd T<br>3rd G |
| 1st A | Ile (I)<br>Met (M) | Thr (T)<br>Thr (T) | Asn (N)<br>Lys (K) | Ser (S)<br>Arg (R) | 3rd T<br>3rd G |
| 1st G | Val (V)<br>Val (V) | Ala (A)<br>Ala (A) | Asp (D)<br>Glu (E) | Gly (G)<br>Gly (G) | 3rd T<br>3rd G |

```
        pIII leader sequence        KpnI

5'- ... TTA TTC GCA ATT CCT TTA GTG GTA CCT TTC TAT TCT CAC TCT
3'- ... AAT AAG CGT TAA GGA AAT CAC CAT GGA AAG ATA AGA GTG AGA

    ... Leu Phe Ala Ile Pro Leu Val Val Pro Phe Tyr Ser His Ser


Start of mature 7 peptide gIII fusion      EagI

NNK NNK NNK NNK NNK NNK NNK GGT GGA GGT TCG GCC GAA ACT GTT GAA
NNM NNM NNM NNM NNM NNM NNM CCA CCT CCA AGC CGG CTT TGA CAA CTT

Xxx Xxx Xxx Xxx Xxx Xxx Xxx Gly Gly Gly Ser Ala Glu Thr Val Glu


AGT TGT TTA GCA AAA TCC CAT ACA GAA AAT TCA TTT ACT AAC ... -3'
TCA ACA AAT CGT TTT AGG GTA TGT CTT TTA AGT AAA TGA TTG ... -5'

Ser Cys Leu Ala Lys Ser His Thr Glu Asn Ser Phe Thr Asn ...

← -28gIII sequencing primer

K = G or T; M = A or C
```

Figure 6. N-terminal sequence of random, seven peptide gIII coat protein fusion (The mature protein starting site is indicated by bold).

remain bound, some with high affinity. Specifically-bound phage remains and is then released under elution conditions. The eluted phage is then amplified in *E. coli,* the enriched phage is collected, and then used to run additional binding/amplification cycles to enrich the pool in a favor of binding sequences. After 3-4 rounds of enrichment, only a few clones remain, the those are characterized by DNA sequencing.

The phage clones are also used for *in vitro* binding affinity assay with the original target. Enzyme-linked immunosorbent assay (ELISA) is widely used for detection of protein binding affinity in the phage display library screening, and the detection level can be as low as $10^{-9}$ g of protein (Hoess *et al*., 1994). The method is to fix the specific target protein on the supporting surface and add the selected binding peptide ligands, *i.e.*, the phage. After short incubating and washing, the unbound phage are washed off. At this point, HRP conjugated anti-M13 antibody is added. The HRP converted colorless OPD substrate into fluorescent yellow-orange color. Only the bound phage can react to anti-M13 antibody and change the substrate color. The color level of the ELISA plate can be read at 492nm.

Short peptide libraries displayed on phage have been used in a number of applications, such as selection of phage-display peptides that bind to cucumber mosaic virus coat protein (Gough *et al*., 1999), differentiating insecticidal activity of soybean cystatins (Koiwa *et al*., 1998), and characterizing acquired resistance in lesion-mimic transgenic potato expressing bacterio-opsin (Abad *et al*., 1997).

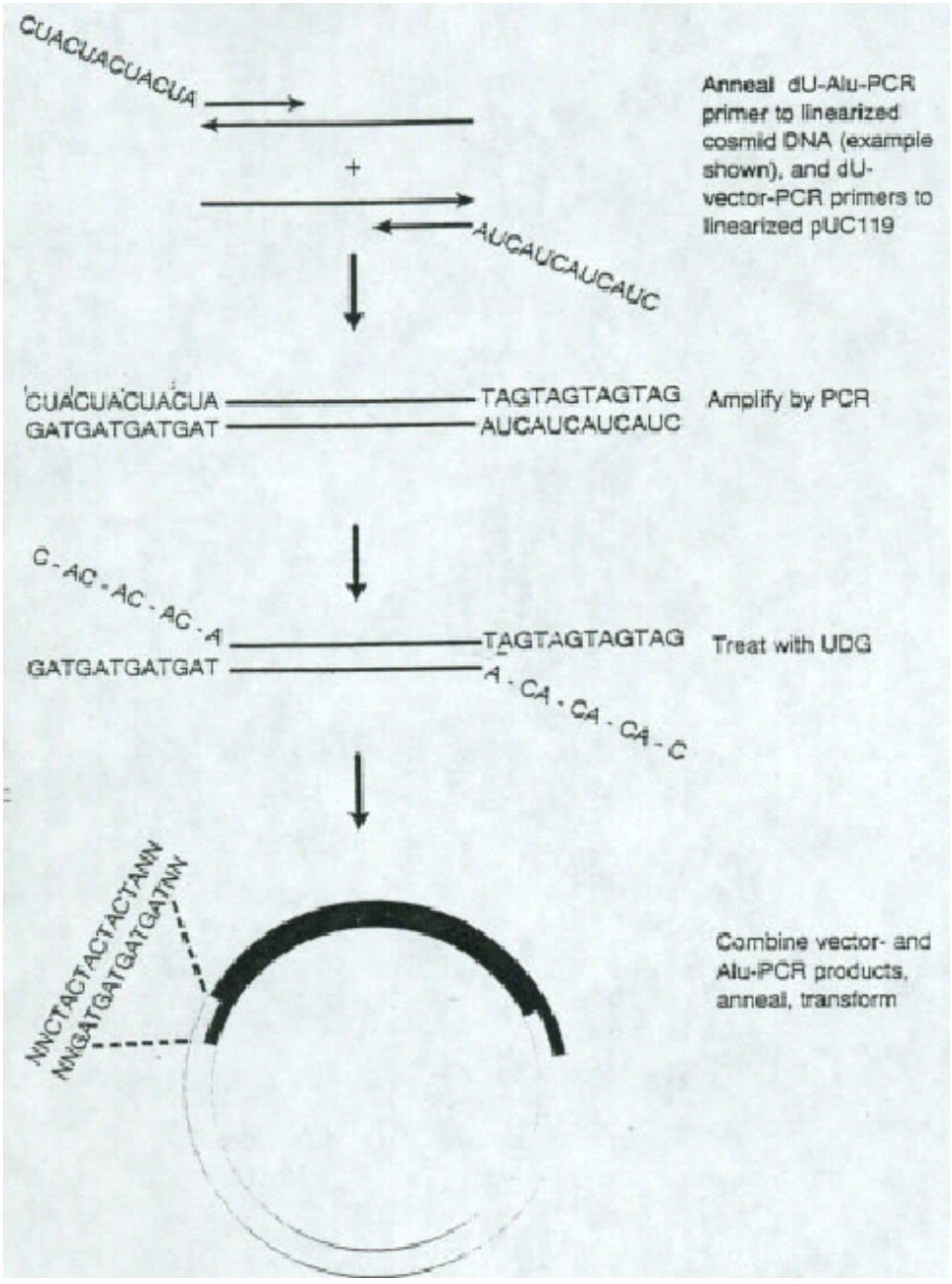



Figure 7. PCR-UDG cloning technique diagram.

## PCR-UDG Cloning Technique

A novel ligation-independent method of site-directed mutagenesis of DNA sequences using PCR amplification followed by uracil DNA glycosylase treatment (termed UDG cloning) has proved to be very efficient and flexible (Rashtchian, 1995). The method is to use two nucleotide primers that contain the desired DNA sequences and which overlap at their 5' ends (Figure 7). The Thymine (dT) residues in the overlap region are substituted with deoxyuracil (dU) in the primer nucleotide sequences. The primers are designed to PCR amplify the entire plasmid to form a linear DNA fragment. The desired base changes are in the primer. The two primers should be complementary to each other (Smith *et al.*, 1993). The presence of dU in the PCR products results the 5' end of the amplified DNA fragments very susceptible to Uracil DNA glycosylase (UDG). The UDG treatment of PCR products leads to excision of dU residues to produce 3' protruding sticky ends. Because the two set of primers are overlapped, the two 3' protruding ends are complementary each other and result in annealing of the PCR products, *i.e.*, the entire plasmid and the mutation sequence of the primer. The circular PCR products are transformed to competent *E. coli* cells and any nicks of circularized plasmid are filled by the *E. coli in vivo* repair system. The new plasmids are identical to the wild-type parental plasmid except for the desired site-directed mutations designed on the primers (Rashtchian *et al.*, 1992).

One research group applied UDG cloning techniques to insert a chloramphenicol-resistance gene into a *Sac*I vector site and three segments of the *lac*Z PCR gene into a

correct orientation (Watson and Bennett, 1997). Another group designed primers with an 11 base overhang as an adaptor to PCR amplify the vector, while the PCR products were treated by UDG to produce overhangs to yield a large numbers of inserts from human cDNA cosmids. This method approved to be a highly efficient way to characterize genes for shotgun sequencing of multiple shorter fragments in a large scale (Andersson *et al*., 1994). Aptamer sequences selected from phage display peptide library biopanning only comprise 21 base pairs (encoding 7 amino acids), and such a short DNA fragment is very difficult to insert into a cloning vector and fuse with other stabilizing reporter genes in the right orientation and in frame. UDG cloning techniques provide good approach to genetic engineering of aptamers into the desired cloning vectors.

## *Agrobacterium tumefaciens* Mediated Transient Expression in Plants

*Agrobacterium tumefaciens* can infect plants and introduce foreign genes into plant cells (Hansen *et al*., 1994). At the site of infection, a crown gall (tumor tissue) forms and synthesizes opines, which are novel amino acids that can only be metabolized by *Agrobacterium* (Cho *et al*., 1997). The tumor is formed, and opines produced, because of genes on the Ti plasmids (tumor-inducing plasmids) carried by *Agrobacterium*. A small portion, usually only a 20kb segment, of the Ti plasmid, called T-DNA (transferred DNA), carries the tumor-inducing and opine-producing genes and integrates into the genome of the infected plant cell (Baron and Zambryski, 1996). The bacteria can infect a susceptible plant only at the site of a wound, where plant cells secret

phenolic compounds that include acetosyringone and help activate the Ti plasmid genes (Chumakov and Kurbanova, 1998). Scientists use molecular cloning techniques to insert foreign DNA fragments between the left and right borders of the T-DNA and use *Agrobacterium* Ti plasmids as a carrier to deliver cloned DNA into plant (Vaquero *et al*., 1999). The ability of *Agrobacterium* T-DNA to integrate stably into its host's genome gives plant molecular biologist a powerful tools to understand gene function. However, transformation of plants take a lot of laboratory effort, and is not successful in every plant species.

Even without stable transformation, *Agrobacterium* Ti plasmids can be used to achieve transient expression of genes in plants. Non-integrated T-DNA copies transiently stay in the nucleus, where they are transcribed (Kapila *et al*., 1997). The transient expression rate is much higher (at least 1,000 times) than that of the stable expression rate in petunia leaf discs (Janssen and Gardner, 1989). Transient gene expression systems are widely used because gene expression can be measured very shortly after T-DNA delivery, there is no position effect, and the gene expression can be tested without regeneration of a transgenic plant (Tai *et al*., 1999). A number of other techniques are used to transfer DNA into intact plant tissue to achieve transient expression, including electroporation (Lindsey and Jones, 1987, Dekeyser *et al*., 1990), particle bombardment (Russell and Fromm, 1995), microinjection (Bilang *et al*., 1993), syringe injection (Robinette and Matthysse, 1990) and vacuum infiltration (Kapila *et al*., 1997). Several *Agrobacterium tumefaciens* host strains such as GV2260 (Deblaere *et al*., 1985), AGL1

(Lazo *et al*., 1991) and LBA4404 (Ooms *et al*., 1982) are commonly used in plant transformation, and in transient expression assays. I used *Agrobacterium tumefaciens* GV2260 with binary vector constructs to inject DNA into citrus, tomato and bean leaves to assay *pthA* gene function and the interactions between the PthA and selected apatmers.

## Experimental Design

The first part of this thesis project was to translationally fused *pthA* with His.Tag, so that the recombinant PthA::His.Tag protein could be purified by His.Bind resin. The COOH terminal 600bp end of *pthA*, encoding all three nuclear localizing signals (NLSs) was translationally fused with GST and cloned into pGEX-4T-3. The recombinant GST fusion protein was purified by Glutathione Sepharose 4B. The two purified recombinant proteins, PthA full length protein and PthA COOH terminal 200 amino acid peptide, were used as the targets to screen the Ph.D.-7 Phage Display Peptide Library to select 7-mer amino acid aptamers by biopanning. The interesting M13 phage clones were DNA sequenced and confirmed to bind PthA by *in vitro* binding affinity assay - ELISA. The aptamer clone sequences were fused to a GUS reporter gene by PCR-UDG cloning. The codon of each amino acid was modified based on the codon preference table of citrus (Table 2). The constructs were then cloned into an *Agrobacterium* binary vector with and without the *pthA* gene and used to inoculate the *Agrobacterium tumefaciens* strains on citrus, bean and tomato leaves. Transient expression assays were used to identity the function of the aptamers in plants.

Table 2. The codon preference table of *Citrus sinensis* (the aptamer coding sequence from phage display biopanning was translated into peptide sequence and replaced by citrus preference codon).

***Citrus sinensis*** [gbpln]: 17 CDS's (6141 codons)

fields: [triplet] [frequency: per thousand] ([number])

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| UUU F 19.5( 120) | UCU S 18.6( 114) | UAU Y 16.8( 103) | UGU C 7.3( 45) |
| UUC F 23.3( 143) | UCC S 13.4( 82) | UAC Y 18.9( 116) | UGC C 10.1( 62) |
| UUA L 11.7( 72) | UCA S 17.4( 107) | UAA * 1.5( 9) | UGA * 0.7( 4) |
| UUG L 19.7( 121) | UCG S 9.0( 55) | UAG * 0.7( 4) | UGG W 12.4( 76) |
| CUU L 23.1( 142) | CCU P 15.0( 92) | CAU H 9.9( 61) | CGU R 5.0( 31) |
| CUC L 16.6( 102) | CCC P 11.7( 72) | CAC H 9.3( 57) | CGC R 4.2( 26) |
| CUA L 6.8( 42) | CCA P 12.5( 77) | CAA Q 18.6( 114) | CGA R 6.0( 37) |
| CUG L 12.7( 78) | CCG P 7.3( 45) | CAG Q 22.0( 135) | CGG R 4.6( 28) |
| AUU I 21.8( 134) | ACU T 20.0( 123) | AAU N 24.8( 152) | AGU S 8.6( 53) |
| AUC I 18.7( 115) | ACC T 15.3( 94) | AAC N 34.5( 212) | AGC S 15.0( 92) |
| AUA I 9.1( 56) | ACA T 18.4( 113) | AAA K 21.2( 130) | AGA R 11.9( 73) |
| AUG M 20.5( 126) | ACG T 10.6( 65) | AAG K 28.8( 177) | AGG R 16.4( 101) |
| GUU V 24.1( 148) | GCU A 32.7( 201) | GAU D 28.7( 176) | GGU G 18.7( 115) |
| GUC V 12.9( 79) | GCC A 20.2( 124) | GAC D 22.0( 135) | GGC G 23.6( 145) |
| GUA V 6.0( 37) | GCA A 19.9( 122) | GAA E 18.4( 113) | GGA G 22.3( 137) |
| GUG V 17.4( 107) | GCG A 9.8( 60) | GAG E 24.8( 152) | GGG G 16.6( 102) |

Coding GC 48.05% 1st letter GC 50.35% 2nd letter GC 43.53% 3rd letter GC 50.28%

# CHAPTER 2
# MATERIALS AND METHODS

## Expression and Purification of PthA Full-length Protein by His.Tag

Full length *pthA* was cloned into the *Bam*HI site of pET-19b to form pYY50.13 (Yang *et al*., 1995) (Figure 8). pYY50.13/*E. coli* DH5α single colonies were inoculated into LB medium (Bacto tryptone 10g/l, Bacto yeast extract 5g/l, NaCl 5g/l, pH 7.5) with 100 μg/ml of ampicillin and grown overnight. DNA mini-preps were made (Sambrook *et al*., 1989) and used to transform *E. coli* protein expression host strain BL21(DE3)pLysS (Studier *et al*., 1990). Fresh pYY50.13/*E. coli* BL21(DE3)pLysS colonies were inoculated into LB medium with 50μg/ml of carbenicillin and 34μg/ml chloramphenicol at 37°C and shaken on a rotary shaker at 220 rpm until the cell density reached $OD_{600}$=0.5. Protein expression was evaluated by using 0.1 mM, 1mM and 100mM IPTG at 28°C with shaking at 220 rpm for 3 hours. The cell pellet was collected and suspended in 1X Binding Buffer (5 mM Imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) plus 0.1% NP-40 and 1 mM PMSF. Cells were then sonicated 60 seconds on ice to disrupt the membranes using a SONICATOR Model W-225 (%duty cycle = 50, out put control=6, pulsed). His.Bind™ resin columns were set up by washing with 3 bed volumes of sterile

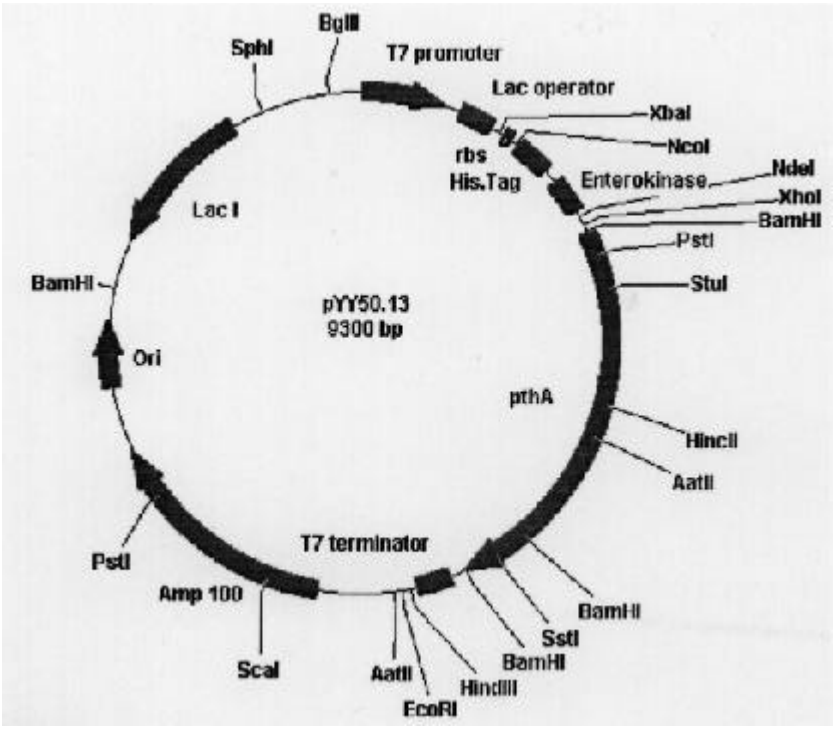



Figure 8. Map of pYY50.13 plasmid (*pthA* gene in pET-19b vector fused with His.Tag).

deionized water, charging with 5 bed volumes of 1X Charge Buffer (50mM $NiSO_4$) and equilibrating with 3 bed volumes of 1X Binding Buffer. His.Tag fusion *pthA* protein crude extract was loaded onto His.Bind resin column. The column was washed by 10 bed volumes of 1X Binding Buffer, 6 bed volumes of 1X Washing Buffer (60mM Imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). His.Tag *pthA* protein was eluted with 1X Elute Buffer (1 M Imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The protein elute was dialyzed against 50 mM $NH_4HCO_3$ at 4°C overnight and lyophilized by

vacuum freeze drying. The freeze dried *pthA* protein was redissolved in 200 µl 1X Treatment Buffer (0.125 M Tris.HCl, pH 6.8, 4% SDS, 20% glycerol, 0.2M DTT, 0.02% Bromophenol Blue, pH 6.8) and examined for purity by SDS polyacrylamide gel electrophoresis. The SDS-PAGE gel was soaked in 0.2 M ice cold KCl and the 130 kDa protein band excised with a sharp razor. The PthA protein was electro-eluted out of the polyacrymide gel slice with Protein Elution Buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS), using a setting of 10mA per tube for 8 hr. The Protein Elution Buffer was exchanged for the same buffer without SDS and electro-elution was continued for another 30 min to remove the SDS from the protein sample. The electro-eluted sample was dialyzed against 50 mM $NH_4HCO_3$ overnight at 4°C and freeze dried. Gel eluted PthA was re-dissolved in 100 µl of 0.1 M $NaHCO_3$ (pH 8.6). The optical density of the sample at 260 nm and 280 nm was measured and used to calculate the protein concentration. The sample was stored at -20°C until ready for use. Details are in Appendix B.

Expression and Purification of GST Fusion PthA C-terminal 200 aa Truncated Protein

The DNA encoding the carboxyl terminal 200 amino acids of PthA was cloned from *pthA* in pYY50.13 by PCR amplification of a 660 bp gene fragment into the pGEX-4T-3 vector (Pharmacia) by Prof. Yingchuan Tian (Gabriel and Tian, unpublished). This resulted in a fusion of the COOH-terminus of PthA to Glutathione S-transferase (GST). The clone was named pGNLS3-2 (Figure 9). Single colonies of pGNLS3-2/*E. coli*

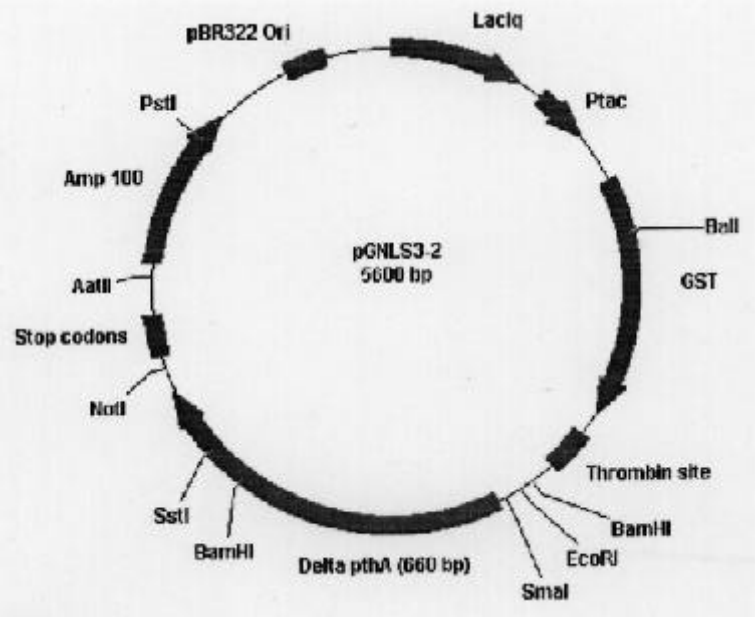



Figure 9. Map of pGNLS3-2 plasmid (*pthA* gene COOH-terminal 660 bp fused with GST in pGEX-4T-3 vector).

DH5α were used to inoculate LB medium with ampicillin to a final concentration of 100μg/ml and mini-preps of pGNLS3-2 plasmid DNA were used to transform *E. coli* protein expression strain BL21(DE3)pLysS competent cells. pGNLS3-2/*E. coli* BL21(DE3)pLysS cells were grown in a rotary shaker at 37°C at 220 rpm in 2X YT medium (Tryptone 16g/l, yeast extract 10g/l, NaCl 5g/l, pH 7.0) with 50μg/ml carbenicillin and 34μg/ml chloramphenicol until growth reached the mid log phage ($OD_{600}$=0.5). Protein expression was induced by addition of 0.1 mM IPTG with continued incubation at 30°C at 220 rpm for 3 hours. Cells were collected by centrifugation at 7,000 rpm at 4°C for 10 min and suspended in 1X PBS (140 mM NaCl,

2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3) plus 0.1% NP-40 and 1 mM PMSF. Cells were disrupted by sonication on ice. The lysate was centrifuged at 12,000 rpm for 20 min at 4°C, the supernatant was collected and passed through a 0.45 micron membrane filter. One ml of this crude protein extract was combined with 100 µl Glutathione Sepharose 4B (a w/v 50% slurry in 1X PBS), and slowly inverted to mix using a rotary platform shaker with gentle agitation at 4°C for 30 min. The GST (Glutathione S-transferase) fusion protein was eluted using 1 ml of Glutathione Elution Buffer (10 mM reduced glutathione, 50mM Tris-HCl, pH 8.0) at 4°C for 30 min. The eluted protein mixture was centrifuged at 500 x g for 10 min at 4°C to pellet the protein. The protein pellet was lyophilized as described previously for PthA. The truncated PthA COOH-terminal 200 aa GST fusion protein was redissolved into 100 µl of 0.1 M $NaHCO_3$ (pH 8.6). Details are in Appendix C.

## Phage Display Peptide Library Biopanning

The full length PthA (His.Tag fusion) and PthA COOH-terminal 200 amino acids truncated protein (GST fusion) were diluted to 100 µg/ml in 0.1 M $NaHCO_3$ (pH 8.6). 100 µg/ml of Glutathione S-transferase in 0.1 M $NaHCO_3$ (pH 8.6) was prepared as pre-absorption blocking agent. Also, 100 µg/ml of Bovine Serum Albumin (BSA) was used as the background control. A 96-well microtiter plate was coated with 150 µl of each target per well, with gentle agitation at 4°C overnight. Each well was rinsed with Blocking Buffer ($NaHCO_3$, pH 8.6, 0.1M, BSA, 5mg/ml, $NaN_3$, 0.02%) for at least 1 hr

at 4°C and then washed 6 times by 1X TBST (Tris.HCl, pH 7.5, 50 mM, NaCl, 150 mM, Tween-20 0.1% v/v) at room temperature. The original unselected library (New England BioLabs, Ph.D.-7$^{TM}$ Phage Display Heptapeptide Library, 2 x $10^{13}$ pfu/ml) was diluted to 2 x $10^{11}$ phage in 1 ml of 1X TBST and incubated on the coated and blocked plates at room temperature for 60 min with gentle agitation.

To reduce non-specific binding of GST fusion protein, the unselected phage library was first incubated with GST coated and blocked plates at room temperature for 60 min, and then transferred to the PthA COOH-terminal 200 aa GST fusion protein coated plates and incubated for another 60 min at room temperature. After incubation, all plates were washed 10 times using 1X TBST. Bound phage was eluted with 100 µl of 100 µg/ml full length PthA protein or PthA COOH-terminal 200 aa truncated GST fusion protein in 1X TBS (Tris.HCl, pH 7.5 50 mM, NaCl, 150 mM) at room temperature for 60 min. The eluted phage was amplified until the titer was up to 2 x $10^{13}$ pfu/ml. These biopanning steps were repeated three times. After the second round of selection, the Tween-20 concentration was increased to 0.5% v/v in the washing steps. For the GST fusion protein, each round of selection used plates that were pre-absorbed by GST. For the PthA full length protein, four rounds of phage display biopanning were carried out. A fourth round of selection was carried out using full length PthA protein on the library previously selected using only the truncated GST fusion PthA COOH-terminal 200 aa protein. At least 10 clones from both screened libraries were picked at random for DNA sequencing. The DNA sequencing primer was -28gIII (5'-

GTATGGGATTTTGCTAAACAAC-3'). The DNA sequencing data was used to find out the concensus sequence of binding clones. Interesting clones were stored at -20°C. Details are in Appendix D.

## *In Vitro* Binding Affinity of Aptamers - ELISA

Representative interesting clones were used for *in vitro* binding affinity assays. The putative phage clone was amplified to a titer of $10^{13}$ pfu/ml. As a control, 1 µl of M13 phage from the original unselected library (New England BioLabs, Ph.D.-7™ Phage Display Heptapeptide Library, 2 x $10^{13}$ pfu/ml) was amplified to a titer of $10^{13}$ pfu/ml. Two targets were used to coat 0.5ml microcentrifuge tubes: 150 µl of 100 µg/ml full length PthA protein in 0.1 M $NaHCO_3$ (pH 8.6) or 150 µl of 100 µg/ml PthA COOH-terminal 200 aa truncated GST fusion protein in 0.1 M $NaHCO_3$ (pH 8.6). As a target background control, 150 µl of 100 µg/ml of BSA in 0.1 M $NaHCO_3$ (pH 8.6) was used. The coated microcentrifuge tubes were incubated with gentle agitation at 4°C overnight. Each tube was then blocked using 200µl Blocking Buffer at 4°C for 2 hr. Serial dilutions of phage in 1X TBST were prepared in a dilution range of $10^7$ to $10^{12}$. Each tube was washed 6 times with 1X TBST at room temperature. To reduce non-specific binding, the serial dilutions of phage were first incubated in microcentrifuge tubes which were only rinsed by Blocking Buffer for 60 min at room temperature and then the pre-absorbed phage was transferred into the blocked target protein tubes to continue incubating at room temperature for another 60 min. Each tube was then washed 6 times with 1X TBS with

0.5% (v/v) Tween-20. Horse Radish Peroxidase/Anti-M13 Monoclonal Conjugate (Pharmacia) was diluted 1:5000 in 1X Blocking Buffer and dispersed 200 µl to each phage-treated tube and incubated at room temperature for 60 min with gentle agitation. Each tube was washed 6 times in 1X TBS with 0.5% (v/v) Tween-20. Sigma FAST OPD$^{TM}$ (O-Phenylenediamine Dihydrochloride) substrate tablet was reconstituted by adding 20 ml distilled water and 200 µl was added to each tube to incubate in the dark at room temperature for 30 min. To stop the reaction, 50 µl of 3M HCl was added to each tube. 150 µl of solution from each tube was transferred to a 96-well ELISA microtiter plate for comparisons. A Microplate Autoreader (model EL 309) Bio-Tek Instruments was used to read absorbance of each well at 492nm. Details are in Appendix E.

## PCR-UDG Cloning of Aptamers

A PCR-UDG cloning strategy was designed to clone the aptamer, YPASYMQ and HPYTFLN, respectively, as the leading sequence and in-frame fusion with GUS reporter gene in a plant expression vector generated from pBI221 (Figure 10). Each amino acid codon was modified based on the citrus codon preference (Table 2). Full length *pthA* gene was cloned into the *Bam*HI site of plant expression vector pBI221 to create a tranlational fusion with GUS reporter gene on the vector by Dr. Yongping Duan (Duan *et al*., 1999). The resulting clone was named pYD12.9F. *E. coli* DH5α was transformed with this vector and single colonies were inoculated into LB medium with ampicillin at a concentration of 100µg/ml and grown at 37°C in a rotary shaker at 220

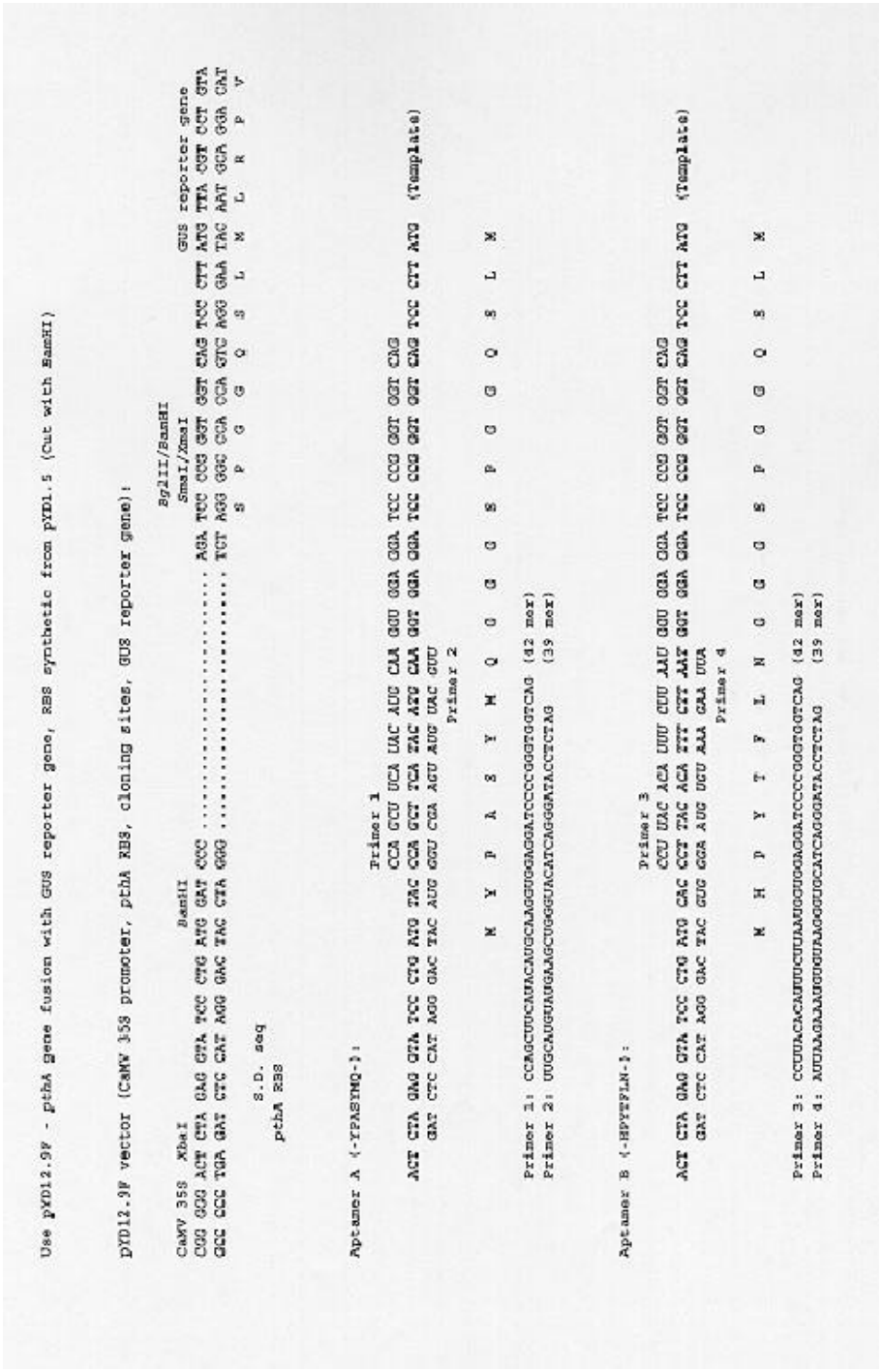



Figure 10. The design of PCR-UDG oligonucleotides to amplify aptamers YPASYMQ and HPYTFLN (The aptamer sequences are indicated by italic on the oligo).

rpm overnight. Plasmid DNA was extracted and digested with *Bam*HI. After phenol/chloroform extraction and ethanol precipitation, pYD12.9F/*Bam*HI fragments were resuspended in TE (pH 8.0) with the final concentration of 5ng/µl. Two sets of oligonucleotides were designed for UDG cloning according to the amino acid sequences of selected aptamers (YPASYMQ and HPYTFLN) and codon preference table of *Citrus sinensis* (Figure 10 and Table 2). Each codon coding the amino acid sequence of aptamers was specifically converted to codon usage appropriate for citrus and the aptamer sequences were included in the synthetic oligonucleotides. Overlaps of 18 nucleotides were designed with 4 to 8 uracil molecules and after Uracil DNA glycosylase treatment, the Uracil residuals dissolved and anneal the overlaps. The oligonucleotides were synthesized by GIBCO BRL and reconstituted in TE (pH 8.0). PCR reaction mixtures were set up as 2µl of 5ng/µl pYD12.9F/*Bam*HI, 15 µl of 10X PCR buffer, 4.5µl of 50 mM $MgCl_2$, 15µl of 2.5mM dNTP, 82.75µl of distilled water. Oligonucleotides were added in two sets: 15 µl each of primers GZ1 (4.36 µM) and GZ2 (4.32 µM) or 15 µl each of primers GZ3 (4.73 µM) and GZ4 (4.82 µM). Details of PCR cycle conditions are in Appendix F. After PCR amplification, Uracil DNA Glycosylase (UDG) treatment was carried out by incubation of 5µl of crude PCR product, 2µl of 10X PCR buffer, 1µl of 1U/µl Uracil DNA glycosylase (GIBCO BRL) and 12 µl of distilled water at 37°C for 30 min followed by denaturation at 65°C for 10 min. The UDG treated PCR products were transformed to *E. coli* DH5α competent cells. Single colonies were inoculated into LB medium with ampicillin at a concentration of 100µg/ml in a rotary shaker at 220 rpm

and grown at 37°C overnight. The plasmid DNA was extracted and then separately digested by *Xba*I/*Bam*HI and *Xba*I/*Sma*I and examined by 15.0% polyacrylamide gel electrophoresis. Interesting clones were saved as glycerol (50% v/v) stock cultures at -70°C. GUS activity assays were carried out by resuspending pellets from 1 ml overnight cultures into 570 µl distilled water and 330 µl of 0.3 mg/m 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). The suspensions were incubated at room temperature overnight. Putative clones showing blue color as compared to the control of *E. coli* DH5α cells were examined further. Plasmid DNA of putative clones was purified using QIAGEN Midi Kits and DNA sequencing was carried out with the DNA sequencing primer DG39R (5'- CATAAGGGACTGACCA -3'). Details are in Appendix F.

## *Agrobacterium tumefaciens* Mediated Transient Expression of Aptamer Constructs in Bean, Sweet Orange and Tomato

Single colonies of above PCR-UDG putative clones were inoculated into LB medium with ampicillin at a concentration of 100µg/ml in a rotary shaker at 220 rpm and grown at 37°C overnight. Plasmid DNA was extracted and digested by *Eco*RI. The digested insert DNA fragments were precipitated by ethanol and redissolved in TE (pH 8.0) to a final concentration of 1µg/µl. Full length *pthA* gene was cloned into pGZ6.4 binary vector by Dr. Yongping Duan, and the resulting clone was named pYD40.1 (Duan *et al*., 1999). As a control, *pthA* was deleted by digesting with *Bam*HI and religation, generated a control clone named pYD40.2 (Duan *et al*., 1999). *E. coli* DH5α/pYD40.1

and DH5α/pYD40.2 were inoculated into LB medium with kanamycin at the concentration of 50μg/ml, respectively, and incubated at 37°C in a rotary shaker at a speed of 220 rpm overnight. Plasmid DNA was extracted and digested by *Eco*RI.

The digested DNA fragments of pYD40.1 and pYD40.2 were separately extracted by phenol/chloroform, precipitated by ethanol, and redissolved in TE (pH 8.0). 20 ng of each *Eco*RI digest were incubated with 5 μl of 10X buffer and 1μl of Shrimp Alkaline Phosphatase (1:1 fresh diluted by 1X Dilution Buffer) in a total volume of 50μl at 37°C for 60 min followed by 65°C denaturation for 20 min. Ligation reactions were set up with 4 μl of *Eco*RI digest of PCR-UDG putative clones, 2μl of the pYD40.1 or pYD40.2 *Eco*RI digests, 2μl of 5X ligation buffer, 1μl of T4 DNA ligase in a total volume of 10 μl at 16°C overnight. 2 μl of the crude ligation mixture was used to transform *E. coli* DH5α competent cells and transformants were selected on LB agar plates containing 50μg/ml of carbenicillin and 50μg/ml of kanamycin.

Single colonies were inoculated into LB medium containing 50μg/ml of carbenicillin and 50μg/ml of kanamycin and incubated in a rotary shaker at 37°C with a speed of 220 rpm overnight. Plasmid DNA extracts were digested by *Eco*RI to confirm the insertion. The orientation of interesting clones was tested by digestion of *Sst*I. Putative clones were mated into *Agrobacterium tumefaciens* GV2260 by tri-parental matings and incubated on LB agar plate at 28°C overnight. Selection for transconjugants was on LB agar plates containing 50μg/ml of carbenicillin, 50μg/ml of kanamycin and 75μg/ml rifomycin. As a control, pYD40.1 and pYD40.2 were also mated into

*Agrobacterium tumefaciens* GV2260 and selected on LB agar plates containing 50μg/ml kanamycin and 75μg/ml rifomycin. Single colonies of *Agrobacterium tumefaciens* GV2260 and transconjugants were inoculated into 20 ml YEB medium (Beef extract 5g/l, yeast extract 1g/l, peptone 5g/l, sucrose 5g/l, $MgSO_4$ 2mM, MES 10mM, acetosyringone 20μM, pH 5.6) with the same antibiotics as used in the mating selections and incubated in a rotary shaker at 28°C and a speed of 60 rpm until the $OD_{600}$ reached 0.8. Bacterial cells were then collected by micro-centrifugation at 4°C and a speed of 5,000 rpm and the pellets were resuspended in MMA medium (1X MS salt, MES 10mM, sucrose 20g/l, acetosyringone 200μM, pH 5.6) until $OD_{600}$=0.5. The suspension was kept at room temperature for 1 hr before use.

Ten-day-old young leaves of California light red kidney bean were infiltrated by *Agrobacterium tumefaciens* GV2260 suspension using vacuum infiltration at a pressure of 0.5 mbar for 20 min. The infiltrated leaves were incubated in 100mm petri-dishes with three-layers of 3MM paper moistured by distilled water and kept at 22°C under light for 36 hr. Also, cell suspensions of *Agrobacterium tumefaciens* GV2260 ($OD_{600}$=0.3) were injected using the blunt end of tubular syringe the abaxial side of tender sweet orange leaves or young tomato leaves (Kapila *et al*., 1997). Inoculated sweet orange plants were grown at 28°C for four weeks in a quarantined green house. The inoculated tomato plants grew under low light for 24 hr in growth chambers. Details are in Appendix G.

# CHAPTER 3
# RESULTS

## Expression and Purification of PthA Protein by His.Tag

Full-length PthA protein expression was induced by adding IPTG. Three different IPTG concentrations (0.1mM , 1mM and 100mM), seven induction times (0hr, 1hr, 2hr, 3hr, 4hr, 5hr and 6hr) and three induction temperatures (28°C, 30°C and 37°C) were tested. The optimum IPTG induction concentration was 0.1mM and the induction temperature was 28°C. 10µl of each sample was mixed with 10 µl of 1X Treatment Buffer (0.125 M Tris.HCl, pH6.8, 4% SDS, 20% glycerol, 0.2M DTT, 0.02% bromophenol blue), boiled 10 min, and centrifuged at 10,000 rpm for 10 min. Supernatant was loaded on 8% (w/v) polyacryamide separating gel with 4% (w/v) stacking gels. Figure 11 shows the time course of full-length PthA protein expression induced by 0.1mM IPTG. A new 130 kDa protein band was very clear after IPTG induction for 1 hr (Figure 11, band D). Maximum induction was obtained after 3hr (Figure 11, band F). Compared to pYY50.13/*E.coli* BL21(DE3)pLysS control (Figure 11, band A) and no IPTG induction control (Figure 11, band B), the results obtained that 0.1mM IPTG was optimum to induce His.Tag fusion protein expression .

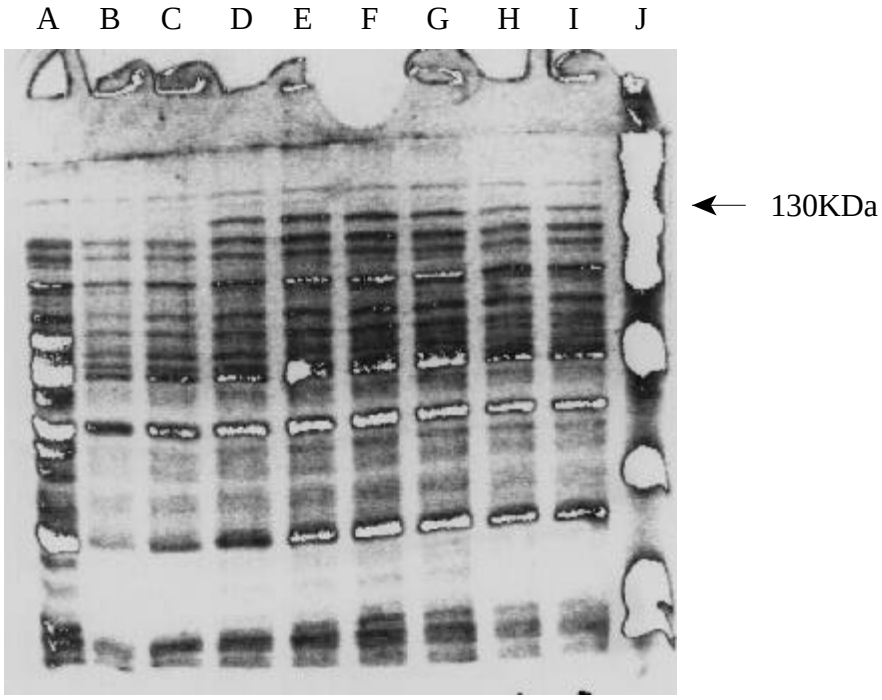



Figure 11. Time course of PthA full length protein expression induced by 0.1 mM IPTG.

A: pYY50.13/*E. coli* BL21(DE3)pLysS, B: no IPTG,
C: IPTG 0 hr, D: IPTG 1 hr, E: IPTG 2 hr, F: IPTG 3 hr,
G: IPTG 4 hr, H: IPTG 5 hr, I: IPTG 6 hr, J: MW marker.

After His.Bind affinity column chromatography, each fraction was collected and 10µl of each sample was mixed 1:1 with 1X Treatment Buffer for SDS-PAGE. Figure 12 shows PthA His.Tag protein after His.Bind affinity column purification. Compared to pYY50.13/*E.coli* BL21(DE3)pLysS control (Figure 12, band A), a good yield of full-length PthA protein was achieved by 0.1m IPTG induction for 3 hr (Figure 12, band B). After sonication, most PthA protein was found in the soluble protein fractions (Figure 12,

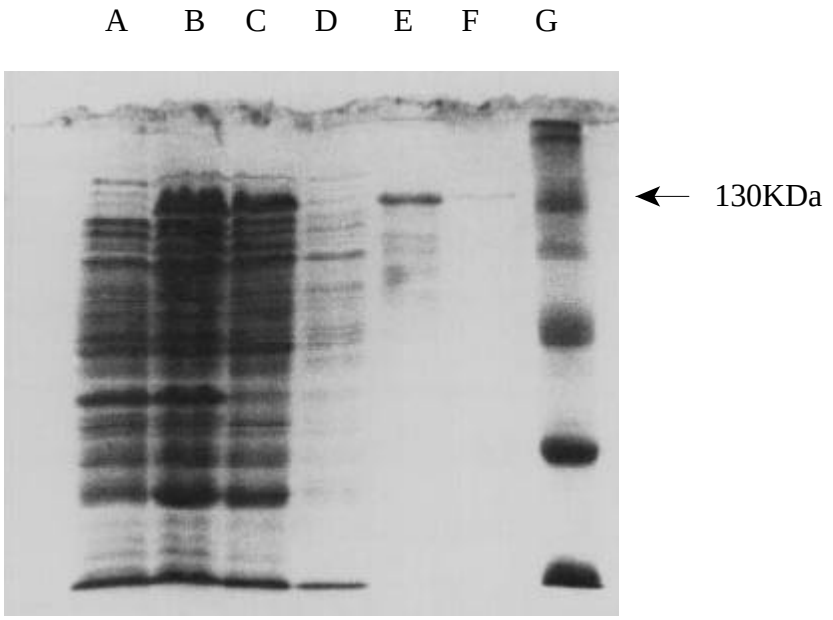



Figure 12. The purification of PthA full length protein by His.Bind™ affinity column chromatography.

A: pYY50.13/BL21(DE3)pLysS no induction, B: 0.1 mM IPTG induction for 3 hr, C: Soluble protein fraction after sonication, D: Soluble protein after His.Bind resin binding, E: His.Bind column PthA protein elute, F: Electro gel elute PthA protein, G: MW marker.

band C). After His.Bind affinity column chromatography, the absence of the 130 kDa band in eluate indicated that most PthA protein was bound to the column (Figure 12, band D). Most of the protein in the elute from the His.Bind column was PthA protein (Figure 12, band E). After electro gel elution, the PthA full-length protein was very pure and appeared as a single clear band at 130 kDa (Figure 12, band F).

Expression and Purification of the PthA COOH-terminal 200aa Truncated Protein by GST Gene Fusion System

Similar experiments were carried out with the PthA COOH-terminal 200aa GST fusion protein. Three different IPTG concentrations (0.1mM , 1mM and 100mM), seven induction times (0hr, 1hr, 2hr, 3hr, 4hr, 5hr and 6hr) and three induction temperatures (28°C, 30°C and 37°C) were tested. The optimum IPTG induction concentration was 0.1 mM and the induction temperature was 30°C.

A B C D E F G H I J

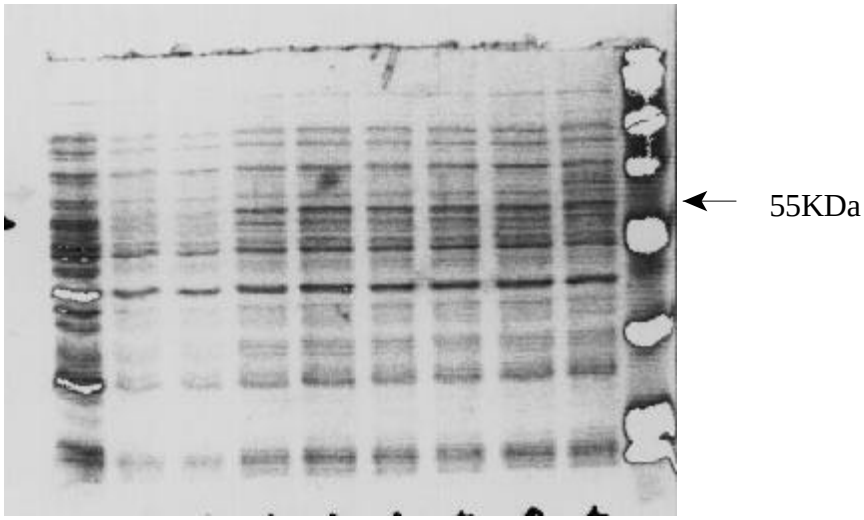



Figure 13. The expression of PthA COOH-terminal 200 aa truncated protein induced by 0.1 mM IPTG at different times.

A: pGNLS3-2/*E. coli* BL21(DE3)pLysS, B: no IPTG, C: IPTG 0 hr,
D: IPTG 1 hr, E: IPTG 2 hr, F: IPTG 3 hr, G: IPTG 4 hr,
H: IPTG 5 hr, I: IPTG 6 hr, J: MW marker.

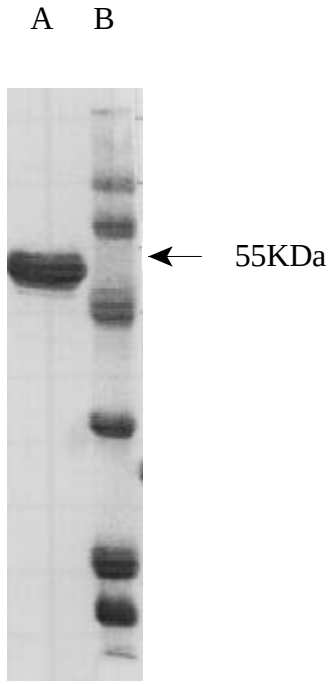



Figure 14. Electro-elution of the COOH-terminal end of PthA, fused to GST.

A: Electro gel elution of PthA C-terminal 200 aa truncated protein, B: MW marker.

Figure 13 shows the expression pattern of GST fusion proteins at 30°C after induction by adding 0.1mM IPTG. After 1 hr of 0.1mM IPTG induction, a new 55kDa protein band appeared (Figure 13, band D) compared to the pGNLS3-2/*E. coli* BL21(DE3)pLysS control (Figure 13, band A), expression of the 55kDa protein reached its peak at 2 hr (Figure 13, band E). Longer induction did not increase the expression level of GST fusion protein (Figure 13, band F-I).

Figure 14 shows the purity of electro-gel-eluted PthA COOH-terminal 200aa GST fusion protein. The GST fusion PthA COOH-terminal 200 aa peptide was found at the expected size of 55 kDa (Figure 14 band A).

## Phage Display Peptide Library Biopanning

After the fourth round of selection, unamplified phage was titered. Single plaques were picked up in plates with about 100 plaques. Single stranded M13 phage DNA was prepared and dissolved in 30µl of TE (pH 8.0) (Appendix D). 1µl of each phage prep was tested by 0.7% agarose gel electrophoresis (Figure 15). Figure 15 Panel A shows 10 randomly selected clones following biopanning selection using PthA full length protein as a target for all four rounds of selection. Figure 15 Panel B shows 10 clones using PthA COOH-terminal 200 aa truncated protein as a target for three rounds of selection and then using PthA full length protein as a target in the $4^{th}$ selection round.

10µl of each selected M13 phage DNA was sequenced and the result is summarized in Figure 16. Of 11 random clones sequenced from the phage population after four rounds of selection, a DNA sequence encoding HPYTFLN appeared 5 times (45.5%), a sequence encoding HPHTFLN appeared twice (18.2%) and a sequence encoding YPASYMQ appeared 4 times (36.4%) (Figure 16, panel A). Of 10 random clones from the phage population after four rounds of selection, a DNA sequence encoding HPYTFLN appeared twice (20.0%) and a sequence encoding YPASYMQ appeared 7 times (70.0%) (Figure 16, panel B).

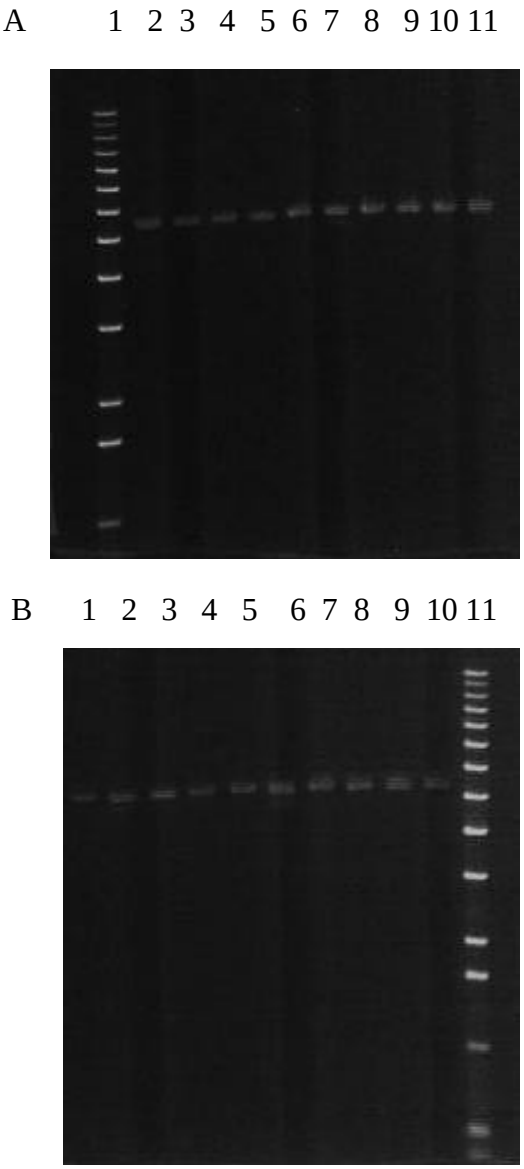



Figure 15. Characterization of M13 PthA-binding clones.

A1:1 Kb DNA Ladder; A2 - A11: 10 clones using PthA full length protein as target in phage display biopanning of all four rounds of selection; B1 - B10: 10 clones using PthA COOH-terminal 200 aa truncated protein as target till 3$^{rd}$ round selection and cross react to PthA full length protein in 4$^{th}$ round selection; B11: 1 Kb DNA Ladder.

A. Phage display sequences of binding clones after 4th round selection (all four rounds of selection against PthA full length protein):

```
  P2.28g HPHTFLN    (ATT CAG AAA CGT ATG CGG ATG)
  P6.28g HPYTFLN    (ATT CAG AAA CGT ATA CGG ATG)
  P7.28g HPYTFLN    (ATT CAG AAA CGT ATA CGG ATG)
  P8.28g HPYTFLN    (ATT CAG AAA CGT ATA CGG ATG)
  P9.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)
 P10.28g HPHTFLN    (ATT CAG AAA CGT ATG CGG ATG)
 P13.28g HPYTFLN    (ATT CAG AAA CGT ATA CGG ATG)
 P14.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)
 P15.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)
 P16.28g HPYTFLN    (ATT CAG AAA CGT ATA CGG ATG)
 P17.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)

         HPYTFLN    (5 of 11, 45.5%)
         HPHTFLN    (2 of 11, 18.2%)
         YPASYMQ    (4 of 11, 36.4%)
```

B. Phage display sequences of binding clones after 4th round cross-reaction against PthA full length protein (previous selection against PthA C-terminal 200 aa truncated protein till 3rd round):

```
  N3.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)
  N4.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)
  N7.28g HYGFPPP    (probably bound to polystyrene well)
  N8.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)
 N11.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)
 N12.28g HPYTFLN    (ATT CAG AAA CGT ATA CGG ATG)
 N13.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)
 N14.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)
 N15.28g HPYTFLN    (ATT CAG AAA CGT ATA CGG ATG)
 N17.28g YPASYMQ    (CTG CAT ATA CGA AGC AGG ATA)

         HPYTFLN    (2 of 10, 20.0%)
         YPASYMQ    (7 of 10, 70.0%)
         Pseudo-    (1 of 10, 10.0%)
```

Figure 16. The DNA sequences of the M13 PthA-binding clones (The conversion of nucleotide sequence to peptide sequence is listed in Table 1).

## *In Vitro* Binding Assay - ELISA

Three representative clones were chosen for ELISA tests, P7.28g (P7) for the HPYTFLN aptamer, N4.28g (N4) for the YPASYMQ aptamer and P10.28g (P10) for the HPHTFLN aptamer. Each clone was amplified until the titer reached $10^{13}$ pfu/ml. The unselected phage from the original phage display library was also amplified until the titer reached $10^{13}$ pfu/ml for use as a control. Serial dilutions of each phage were prepared with titers ranging from $10^{12}$ pfu/ml to $10^{7}$ pfu/ml. The target control was Bovine Serum Albumin (BSA). Figure 17 shows a 96-well ELISA plate loaded with 150 μl of reaction mixture, which was pipetted out of 0.5 ml microcentrifuge tubes and read at 492nm using a Microplate Autoreader EL309. There was no reaction between phage (unselected original M13 phage(WT), HPYTFLN aptamer (P7), YPASYMQ aptamer (N4) or HPHTFLN aptamer (P10)) and BSA, the "pseudo-target". There also was no reaction between unselected original M13 phage (WT) and PthA full-length protein (pthA) or the COOH-terminal 200 aa truncated PthA GST fusion protein (NLS). HPYTFLN aptamer (P7), YPASYMQ aptamer (N4) and HPHTFLN aptamer (P10) all reacted to PthA full-length protein (pthA) and to the COOH-terminal 200 aa truncated PthA GST fusion protein (NLS). The reaction of the aptamers to the full length PthA protein looked stronger than to the truncated protein. Figure 18 is the summary of ELISA data read at 492nm. The result indicated that HPYTFLN (P7), YPASYMQ (N4) and HPHTFLN (P10) aptamer phage all had strong binding affinity to PthA full-length protein and the

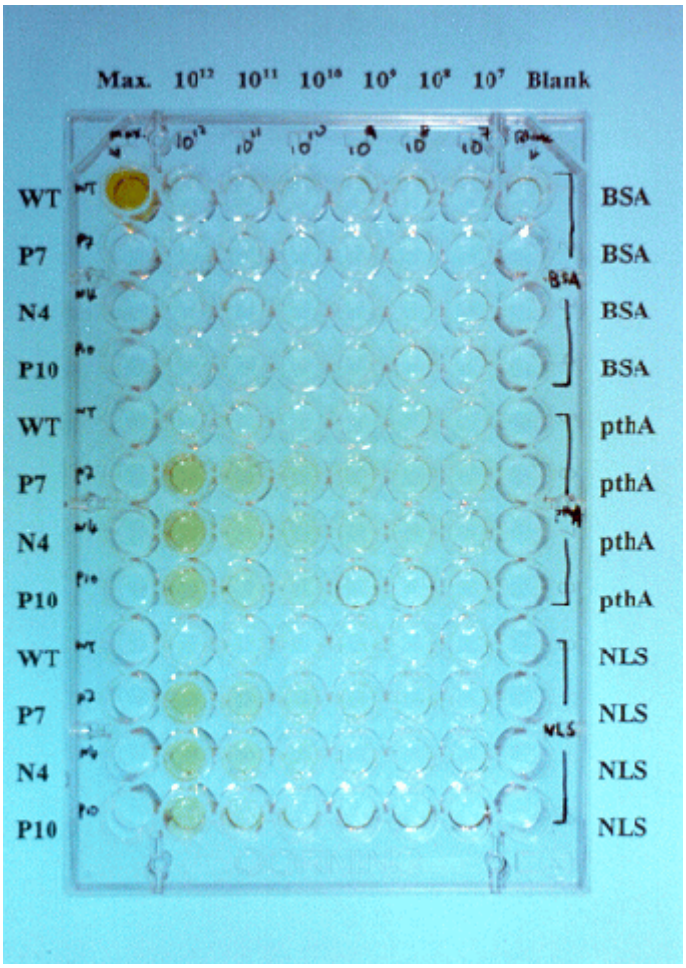



Figure 17. *In vitro* Binding Assay of Aptamers.

ELISA Test for different concentrations of WT (no selective random phage), P7 (HPYTFLN phage), N4 (YPASYMQ phage) and P10 (HPHTFLN phage) against different targets including BSA (Bovine Serum Albumin), pthA (PthA full length protein) and NLS (PthA COOH-terminal 200 aa truncated protein).

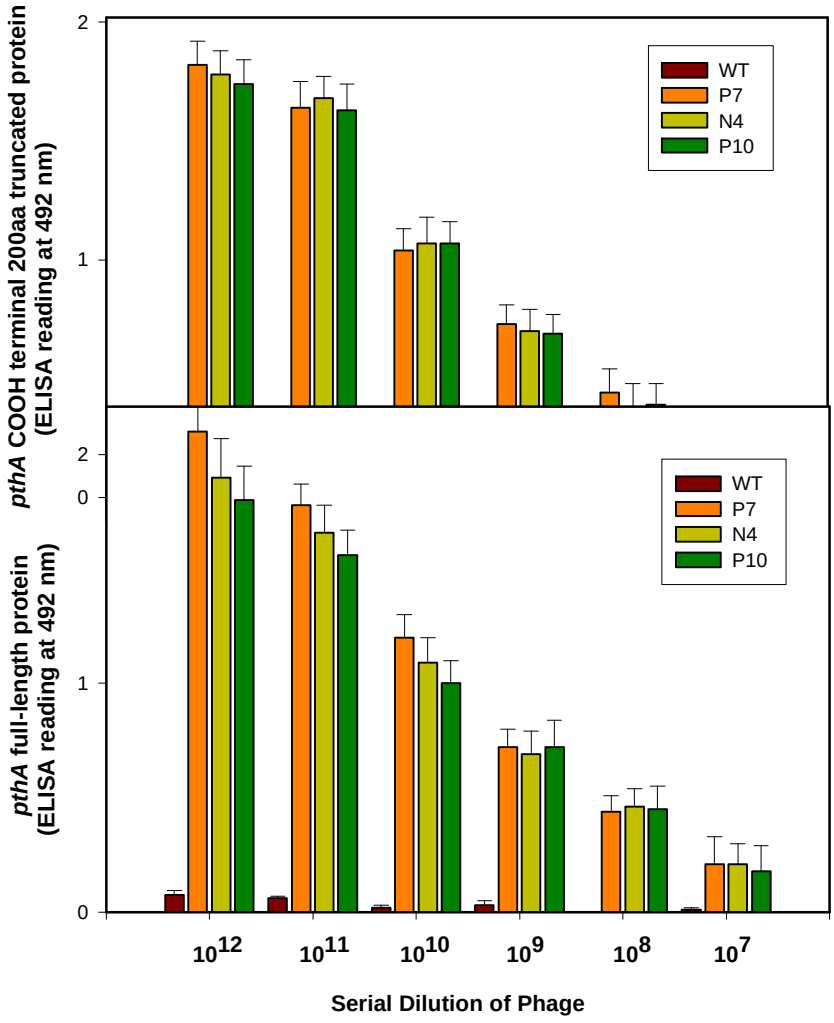



Figure 18. The *in vitro binding* afnity of aptamers: P7 (HPYTFLN), N4 (YPASYMQ) and P10 (HPHTFLN).

COOH-terminal 200 aa truncated PthA protein. The binding affinity diminished with dilution. There was not much difference in binding affinity among the three different aptamers.

PCR-UDG Cloning of Aptamers

Two aptamer sequences, encoding YPASYMQ and HPYTFLN, were synthesized and amplified by PCR for UDG-cloning into pBI221 using the pYD12.9F/*Bam*HI fragment as the template (Figure 19, band C). PCR products were tested by 0.7% agarose gel electrophoresis. Figure 19 band E shows the GZ1 and GZ2 primer amplified PCR products with the sequence of YPASYMQ and Figure 19 band G shows the GZ3 and GZ4 primer amplified PCR products with the sequence of HPYTFLN. Figure 19 band D and band F are negative controls without template. The PCR products were at the expected size of 5.8 kb. After Uracil DNA glycosylase treatment, the linear PCR products were reclosed (Figure 20). Further testing by double restriction enzyme digestion yielded the expected aptamer size of 45 base pairs between the *Xba*I site and the *Bam*HI sites (Figure 10). Figure 21 indicates that both pGZ7.5 and pGZ7.6 have 45 bp insertions (Figure 21, band D and band F). The 90 bp band is likely an aptamer dimer. The insertion sequence was confirmed by DNA sequencing. Two clones were saved for further testing . The pGZ7.5 for the YPASYMQ apatmer (Figure 20 band F) and pGZ7.6 for the HPYTFLN aptamer (Figure 20 band M). Maps of pGZ7.5 and pGZ7.6 are shown in Figure 22.

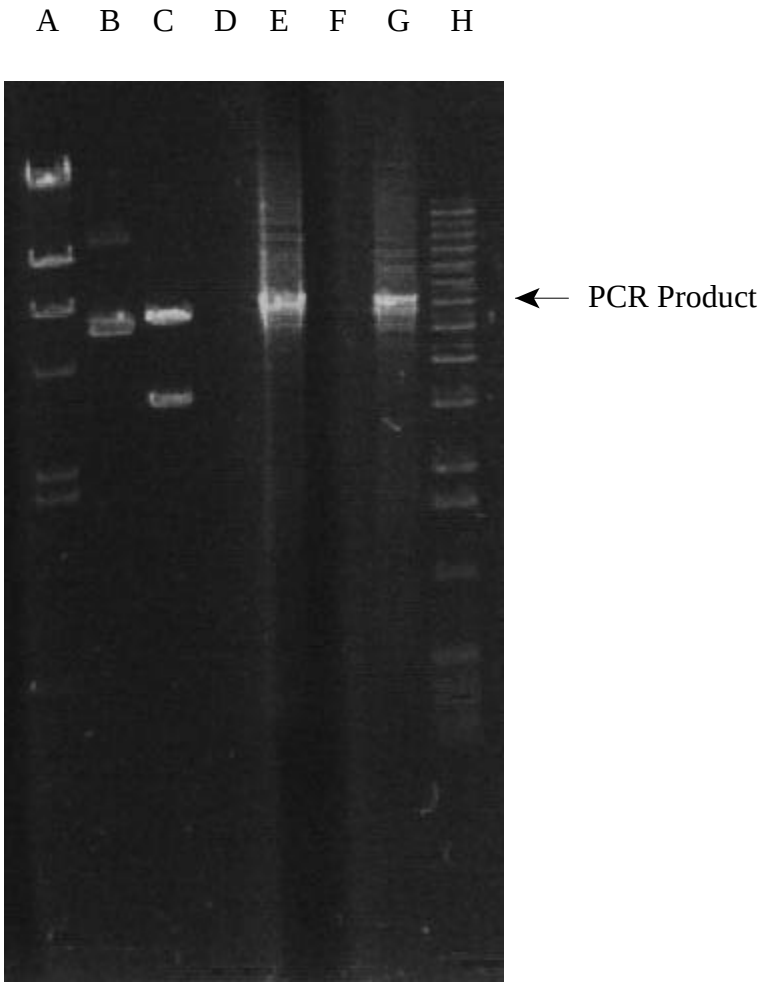



Figure 19. PCR amplification of aptamers P7 and N4.

A: λDNA/*Hind*III fragments; B: pYD12.9F plasmid DNA, C: pYD12.9F/*Bam*HI template, D: pGZ1 primer + pGZ2 primer (no template), E: pGZ1 primer + pGZ2 primer + pYD12.9F/*Bam*HI template, F: pGZ3 primer + pGZ4 primer (no template), G: pGZ3 primer + pGZ4 primer + pYD12.9F/*Bam*HI template, H: 1 Kb DNA Ladder.

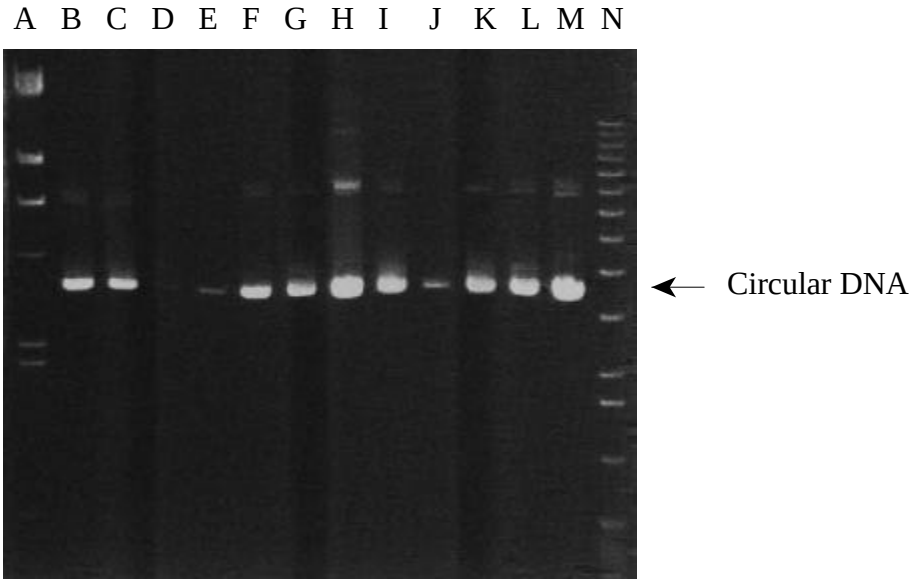



Figure 20. Uracil DNA glycosylase treatment of PCR products.

A: λDNA/*Hind*III fragments, B to G: pGZ1 primer and pGZ2 primer amplified "YPASYMQ" clones (F clone saved as pGZ7.5), H to M: pGZ3 primer and pGZ4 primer amplified "HPYTFLN" clones (M clone saved as pGZ7.6), N: 1 kb DNA Ladder.

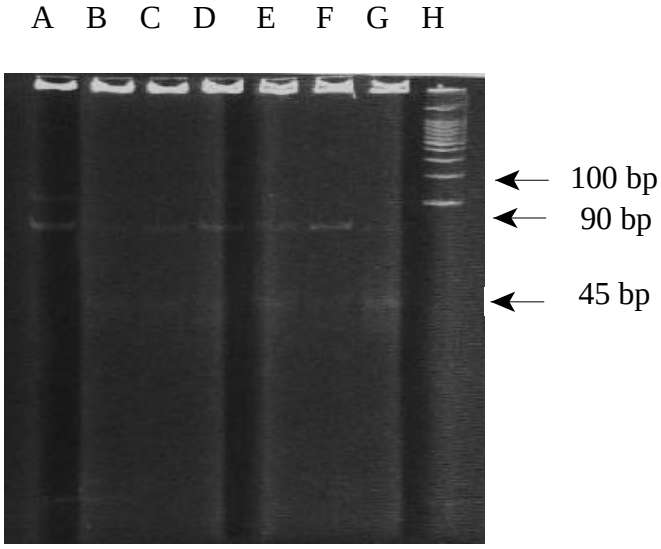
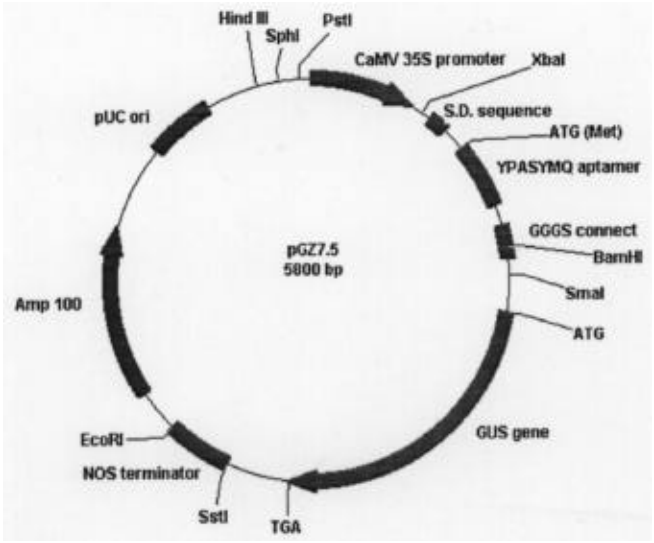



Figure 21. 15.0% polyacrylamide gel electrophoresis of apatmers.

A to C: Other PCR-UDG clones digested by *Xba*I+*Bam*HI,
D: pGZ7.5+*Xba*I+*Bam*HI, E: pGZ7.5+*Xba*I+*Sma*I, F: pGZ7.6+*Xba*I+*Bam*HI,
G: pGZ7.6+*Xba*I+*Sma*I, H: 100 bp DNA Ladder.



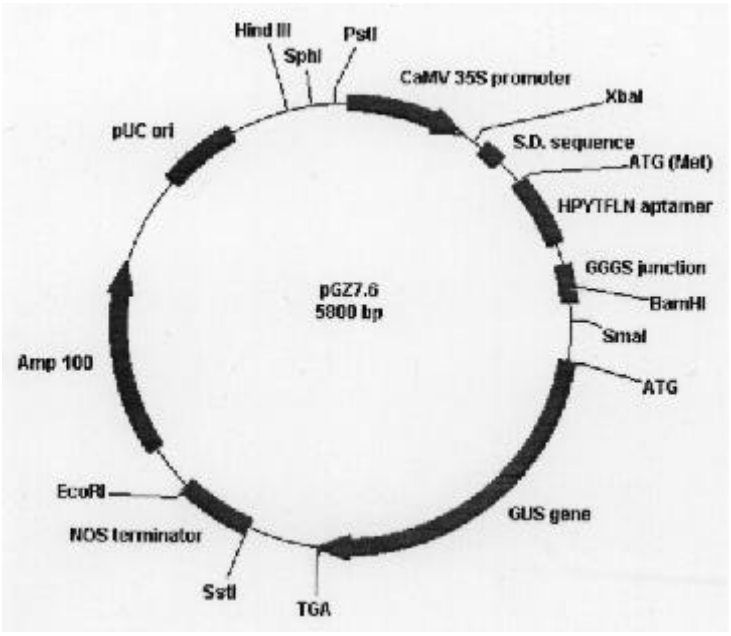




Figure 22. Plasmid maps of pGZ7.5 and pGZ7.6.

## *Agrobacterium tumefaciens* Mediated Transient Expression of Aptamers in Bean, Sweet Orange and Tomato Leaves

Constructs of pGZ7.5 and pGZ7.6 cloned into *Agrobacterium* binary vector (pYD40.1 and pYD40.2) are shown in Figure 23. *Eco*RI digestion confirmed the size of insertion and *Sst*I digestion indicated the orientation of inserted fragments. Four clones were saved: pGZ8.1 carries the coding sequence for the YPASYMQ aptamer and *pthA* (Figure 23, band 2), pGZ8.2 carries the coding sequence for the YPASYMQ aptamer (Figure 23, band 17), pGZ8.3 carries the coding sequence for the HPYTFLN aptamer and *pthA* (Figure 23, band 28) and pGZ8.4 carries the coding sequence for the HPYTFLN aptamer (Figure 23, band 31). Maps of these four clones shown in Figure 24.

Figure 25 Panel A shows *Agrobacterium tumefaciens* GV2260 constructs after infiltration into young California light red kidney bean leaves. Compared to the dark brown Hypersensitive Reaction (HR) lesion of the positive control from pYD40.1 (expressing *pthA*), pGZ8.1 (expressing the YPASYMQ aptamer and *pthA*) and pGZ8.3 (expressing HPYTFLN aptamer and *pthA*) showed reduced HR symptoms. pGZ8.1 showed significantly reduced HR, indicating that the YPASYMQ aptamer may work better in plant than the HPYTFLN aptamer.

Figure 25 Panel B shows *Agrobacterium tumefaciens* GV2260 constructs inoculated into young sweet orange leaves. Compared to the strong canker symptom expressed by pYD40.1 (*pthA*), pGZ8.1 (expressing the YPASYMQ aptamer and *pthA*)

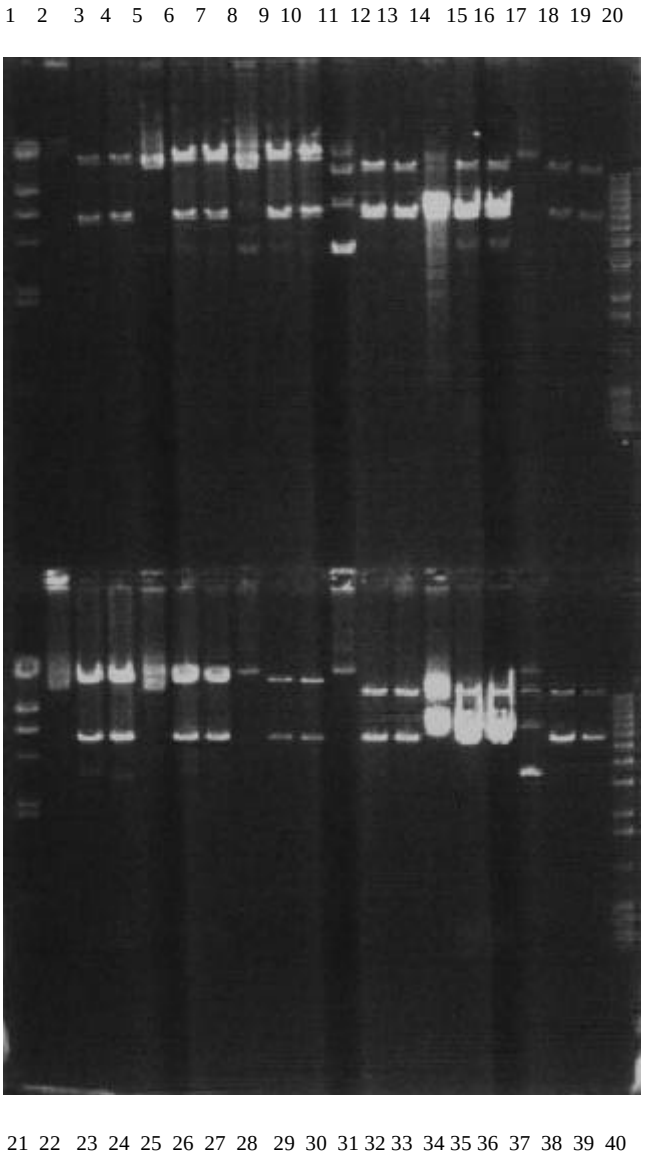



Figure 23. Aptamers P7 and N4 cloned in *Agrobacterium* binary vector pYD40.1 and pYD40.2.

1, 21: λDNA/*Hind*III fragments; 20, 40: 1 kb DNA Ladder; 2 to 10: pYD40.1+pGZ7.5; 11 to 19: pYD40.2+pGZ7.5; 22 to 30: pYD40.1+pGZ7.6; 31 to 39: pYD40.2+pGZ7.6. Each group has three different clones. Each clone has three different treatments, such as undigested plasmid DNA, digested by *Eco*RI and digested by *Sst*I. (Save No.2 clone as pGZ8.1, No.17 clone as pGZ8.2, No.28 clone as pGZ8.3, and No.31 clone as pGZ8.4, respectively).

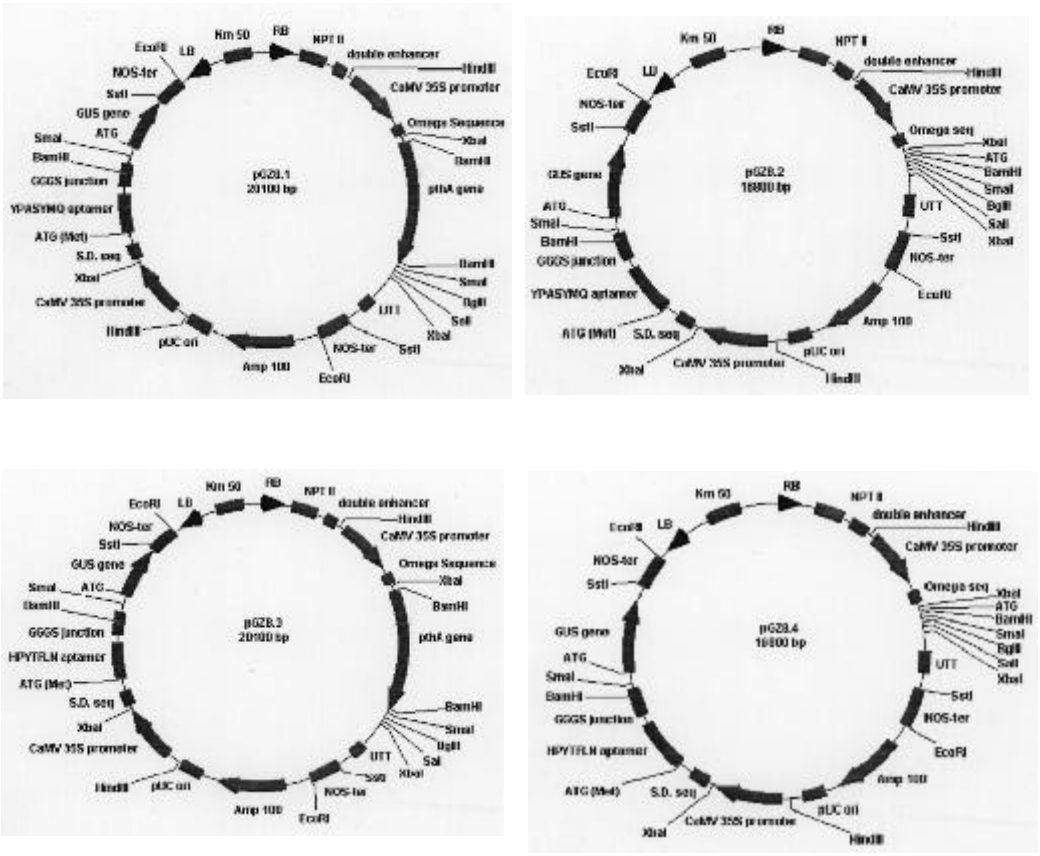
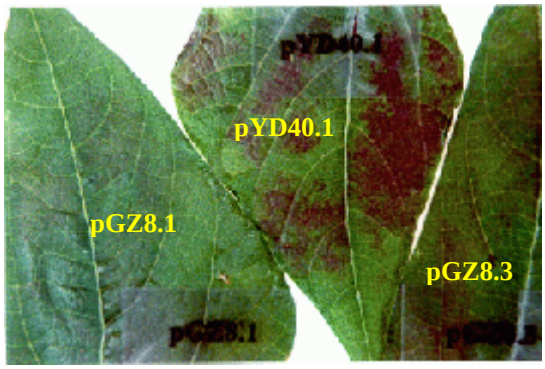
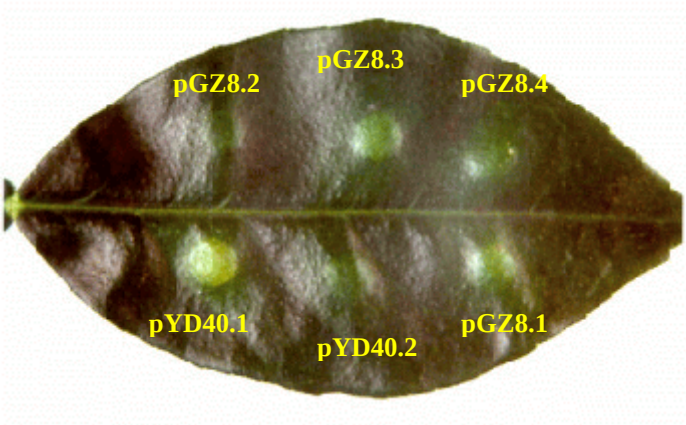



Figure 24. The plasmid maps of pGZ8.1, pGZ8.2, pGZ8.3 and pGZ8.4.

A. Bean




B. Citrus




C. Tomato

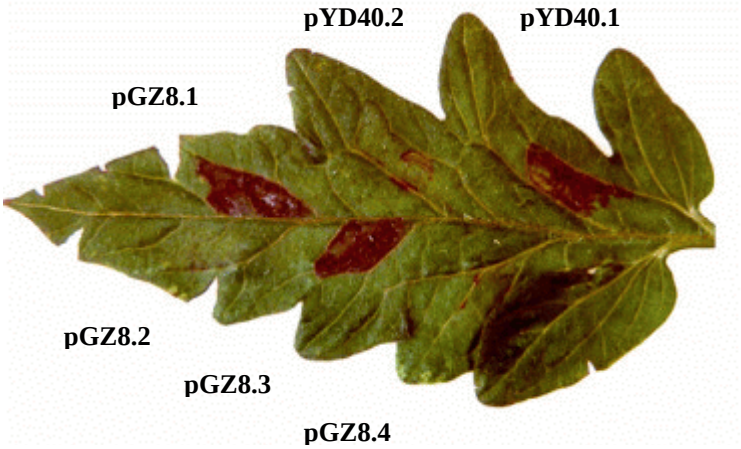



Figure 25. Inoculation of *Agrobacterium tumefaciens* strain GV2260 constructs to the leaves of bean (panel A), citrus (panel B) and tomato (panel C).

pYD40.1 is *pthA* gene only in binary vector, pYD40.2 is the binary vector, pGZ8.1 is YPASYMQ aptamer sequence with *pthA* gene in binary vector, pGZ8.2 is YPASYMQ aptamer sequence only in binary vector, pGZ8.3 is HPYTFLN aptamer sequence with *pthA* gene in binary vector and pGZ8.4 is HPYTFLN aptamer sequence only in vector.

and pGZ8.3 (expressing the HPYTFLN aptamer and *pthA*) showed reduced canker symptoms. Also, pGZ8.1 reduced canker symptoms more than pGZ8.3, which confirmed the observations in bean. pYD40.2 (binary vector only), pGZ8.2 (expressing the YPASYMQ aptamer only) and pGZ8.4 (expressing the HPYTFLN aptamer only) showed no effects on any plants tested.

Figure 25 Panel C shows inoculation of tomato young leaves using the same *Agrobacterium* constructs. There was no difference in HR symptoms between pYD40.1 (expressing *pthA*), pGZ8.1 (expressing the YPASYMQ aptamer and *pthA*) and pGZ8.3 (expressing the HPYTFLN aptamer and *pthA*). This result indicated that the two aptamers had no effect on HR expression in tomato, as expected. Published reports and our own lab results (Gabriel *et al*., 1996) showed that the COOH-terminal region of members of the *pthA* gene family are not important for elicitation of the HR in tomato. The most plausible explanation for this results is that both the YPASYMQ aptamer and the HPYTFLN aptamer bind to the COOH-terminal region of PthA protein, thus, it didn't show the effect of block PthA function.

# CHAPTER 4
# DISCUSSION

A Phage Display Peptide Library was used to screen for 7-peptide aptamers that specifically bind to PthA full-length protein and a PthA C-terminal 200aa truncated protein. Two recombinant protein were constructed, *pthA* full length gene in pET-19b vector for His.Tag fusion and pthA COOH terminal 600bp gene fragment in pGEX-4T-3 vector for GST fusion.

The His.Tag fusion protein was best expressed by vigorously shaking at 37°C followed by 0.1mM IPTG induction at $OD_{600}$=0.5, changing the temperature to 28°C for 3 hr. The best condition for GST fusion protein expression was vigorously shaking at 37°C in 2X YT medium till $OD_{600}$=0.5, followed by adding of 0.1mM IPTG and shaking at 30°C for 3 hr. The low concentration of IPTG and lower temperature induction appeared to make the fusion proteins more soluble and more easily purified by resin affinity purification. Use of *E. coli* BL21(DE3)pLysS as a host strain significantly enhanced the expression rate of the fusion protein compared to non-host *E. coli* strain DH5α. Also, since Ampicillin and Chloramphenicol had a synergistic effect, Carbenicillin was used to replace Ampicillin in combination with chloramphenicol for

antibiotic selection. Proteinase inhibitors such as PMSF appeared to inhibit proteinase activity during sonication and protected the fusion protein from degradation. Nonionic detergents such like NP-40 increased the solubility of fusion proteins. All reactions were kept on ice to obtain the maximum yield of fusion proteins. Quick sonication on ice helped keep a high protein yield and high-speed centrifugation was used to collect soluble proteins. For His.Tag protein purification, the His.Bind resin needed to be regenerated before use and well washed, charged and equilibrated. Such treatment significantly enhanced the efficiency of the affinity column. A flow rate of about 10 column volumes per hour was required and if necessary, the pass-through was collected and reloaded on top of the column and run again to maximize the binding of His.Tag protein to the column. The His.Tag protein always come out in the first 5 ml of eluate. Later eluate contained other proteins with some affinity to the His.bind resin. Protein was dialyzed and redissovled in as small a volume as possible to increase the concentration.

For GST fusion protein, low speed (500xg) centrifugation was necessary to sediment the Glutathione Sepharose 4B without compacting the pellet. Gentle agitation during elution was required. The Glutathione Elution Buffer was freshly made to keep it in a reduced state. Dialysis against 50mM $NH_4HCO_3$ is one of the modifications used for easy purification of lyophilized proteins. After freeze drying, the $NH_4HCO_3$ evaporated and the protein sample was dissolved in any desired salt solutions without changing of ion strength. After eight hours of electro elution at 10mA per tube, the Protein Elution Buffer without SDS was continued another 30 min.

There were two target proteins in Phage display biopanning, PthA full-length His.Tag protein and PthA COOH-terminal 200aa truncated GST fusion protein. Because the pET-19b vector only had 10 Histidine repeats, compared to the size of PthA full-length protein (130 kDa), the His.Tag region was very small. Therefore, PthA full length His.Tag protein could be directly used as the target for phage display biopanning. But for the GST fusion protein, the Glutathione S-transferase itself has a molecular weight of 26 kDa, and the size of PthA COOH-terminal 200 aa peptide was 29 kDa (Figure 3, bold letters). In this case, Glutathione S-transferase could readily interact with the truncated PthA peptide and have some side effect. Therefore, the Glutathione S-transferase could be separated using a cleavage enzyme and the PthA COOH-terminal peptide could be recovered by gel purification. In this experiment, during phage display biopanning, the amplified phage was first incubated with pure Glutathione S-transferase. Any phage bound to GST was eliminated during this pre-absorbing treatment, then, unbounded phage was applied to against GST fusion protein in biopanning. After three rounds of separate selection, a fourth round of cross-selection using full length PthA was carried out. For PthA full length protein biopanning, PthA full-length protein was used as the target in all selections. Three different aptamer encoding sequences were found: YPASYMQ, HPYTFLN and HPHTFLN. YPASYMQ was found 4 times out of 11 (36.4%), HPYTFLN was found 5 times out of 11 (45.5%) and HPHTFLN was found 2 times out of 11 (18.2%). In the phage population from selection against the PthA COOH-terminal 200 aa truncated GST fusion protein, YPASYMQ was found 7 times out of 10 (70.0%) and HPYTFLN was found 2 times out of 10 (20.0%). No HPHTFLN

sequence was found in this case. Based on previous reports (Hoess et al., 1994), some phage containing sequences with repeats of Phenylalanine or Histidine especially could bind to polystyrene well. One such sequence, encoding the sequence of HYGFPPP, wass considered to be a pseudo-binding sequence. Based on the *in vitro* binding affinity assay, ELISA readings indicated HPYTFLN and HPHTFLN had very similar reactivity both to PthA full length protein and PthA COOH-terminal 200 aa truncated GST fusion protein, so HPHTFLN could be considered as the subgroup of HPYTFLN with only one base pair change in the DNA sequence. It is interesting that there were only two aptamer sequences found in two independent selective pathways, and that they were equally distributed within two selection phage populations.

*In vitro* binding affinity assay was carried out by ELISA reading at 492 nm. The optimal reading value should be in the range of 0.5 to 2.0. M13 phage itself may bind to ELISA plates. M13 phage always showed high background readings and it was very hard to tell the difference between the phage binding to plate or the phage binding to target. After a couple of trials, I found that 0.5 ml microcentrifuge tubes (usually used for PCR reaction) were very good for reducing the background of M13 in ELISA reactions. To reduce background, one set of tubes were blocked by Blocking Buffer. The phage was first put into only blocked empty tubes to incubate for 60 min at room temperature, then, pre-absorbed phage was transferred to the tubes with blocked target. This step helped to eliminate non-specific phage only binding to the tube surface. The unbound phage was used in binding to the target. As a target control, BSA was used. YPASYMQ and HPYTFLN didn't bind to BSA. Also, as a phage control, original unselected phage was

used. The ELISA result showed that unselected phage didn't bind to PthA full length protein or PthA COOH-terminal 200 aa truncated GST fusion protein. Since the original library complexity is 2 x $10^{13}$ pfu/ml, the frequency of two aptamer sequences in the unselected original phage population is almost zero, the reaction of unselected original phage could be considered as the background. In the ELISA results, aptamers bound to PthA full length protein with higher affinity than bound to PthA COOH-terminal 200 aa truncated GST fusion protein. This indicated that the aptamers interacted more with the full-length PthA protein.

PCR amplification was carried out by a program called "touch-down". Based on the *Tm* of primers (GZ1 at 58°C, GZ2 at 54°C, GZ3 at 58°C and GZ4 at 54°C), a program was designed with annealing temperatures ranging from 56°C to 50°C, dropping one degree of centigrade once in a total of seven respective cycles, followed by a regular 30 cycles of PCR carried out at 94°C for 1 min, 65°C for 1 min and 72°C for 5 min. Elongation times were determined by the length of the expected PCR amplified molecules. For an expected PCR product of 5.8 Kb, elongation time was set up at 5 min. Uracil DNA glycosylase was used to remove Uracil. The intervening bases was then melted off at 65°C, and the 3' overhangs reannealed (See Figure 10). The aptamers in pBI221 were engineered to form the peptides of sequence M-YPASYMQ-GGGS-PGGQSLM-GUS and M-HPYTFLN-GGGS-PGGQSLM-GUS. The starting methionine seemed to have no effect on aptamer function. The Glu-Glu-Glu-Ser forms a flexible arm to ensure that the aptamer peptide protrudes out of the protein fusion complex. The GUS protein provides both stability to the aptamer and is useful for detection of plant

transformation. The aptamer fusion sequence was given a CaMV35S promoter and terminated by a NOS terminator, so it could be used in plant expression (Figure 22).

The two aptamer constructs were cloned into the *Agrobacterium* binary vector pYD40.1 (with *pthA*) and pYD 40.2 (vector only), and mated into *Agrobacterium tumefaciens* GV2260 strains, then vacuum infiltrated into California light-red kidney bean young leaves and syringe inoculated into sweet orange and tomato leaves. pYD40.1 (*pthA* alone) showed very strong Hypersensitive Reaction (HR) brown lesions on California light red kidney bean leaves, while pGZ8.1 (YPASYMQ aptamer with *pthA*) and pGZ8.3 (HPYTFLN aptamer with *pthA*) showed significantly reduced HR symptoms. The YPASYMQ aptamer appeared to work better than the HPYTFLN aptamer. A similar result also showed in sweet orange inoculations. Compared to the strong canker symptom of pYD40.1 (*pthA* alone), the YPASYMQ aptamer with *pthA* and the HPYTFLN aptamer with *pthA* showed reduced canker symptoms. Also, the YPASYMQ aptamer reduced canker symptoms more than the HPYTFLN aptamer, which confirmed the observations in bean. The aptamers alone, when expressed in plant cells, showed no effect. Inoculating tomato showed there is no difference between *pthA* alone or *pthA* with aptamer which indicated the aptamer may interact the COOH terminal of *pthA* gene family.

Figure 26 shows the predicted antigen index of PthA full-length protein. Figure 27 shows the predicted KD hydrophilicity and KD hydrophobicity of PthA full-length protein. Figure 28 shows the predicted surface probability of PthA full-length protein.

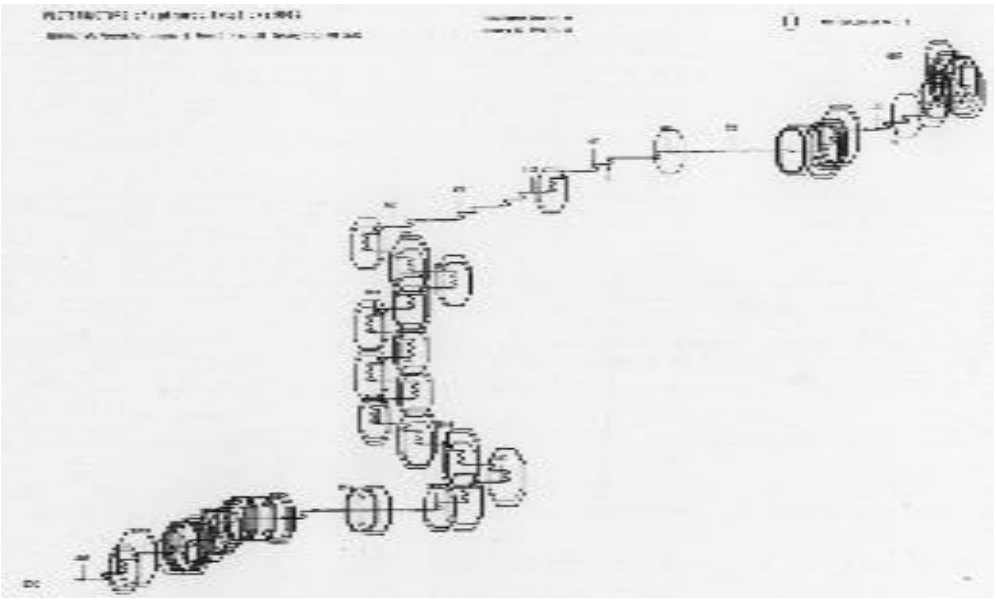


Figure 26. The predicted Antigenicity Index of PthA.

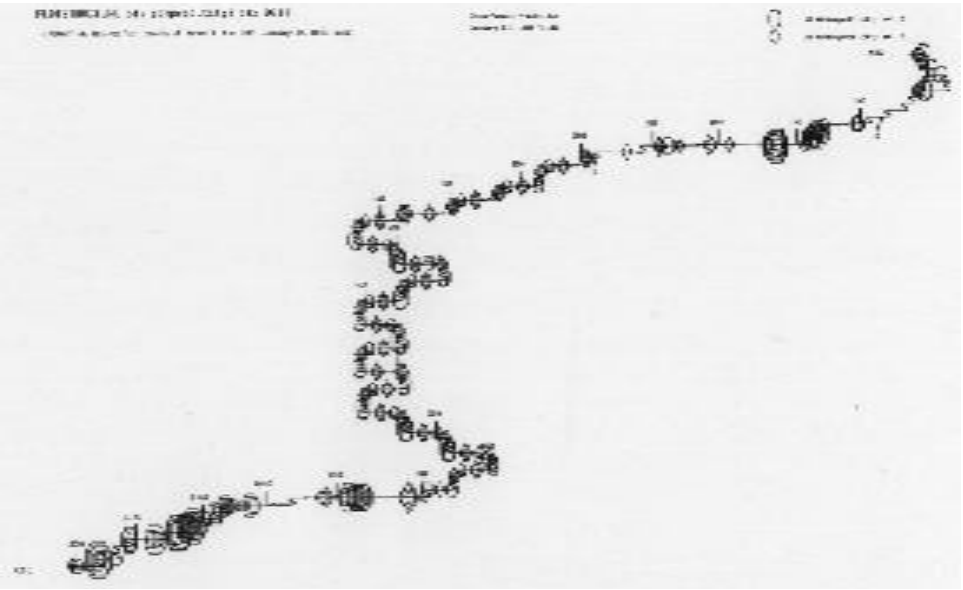


Figure 27. The predicted KD Hydrophilicity and KD Hydrophobicity of PthA.

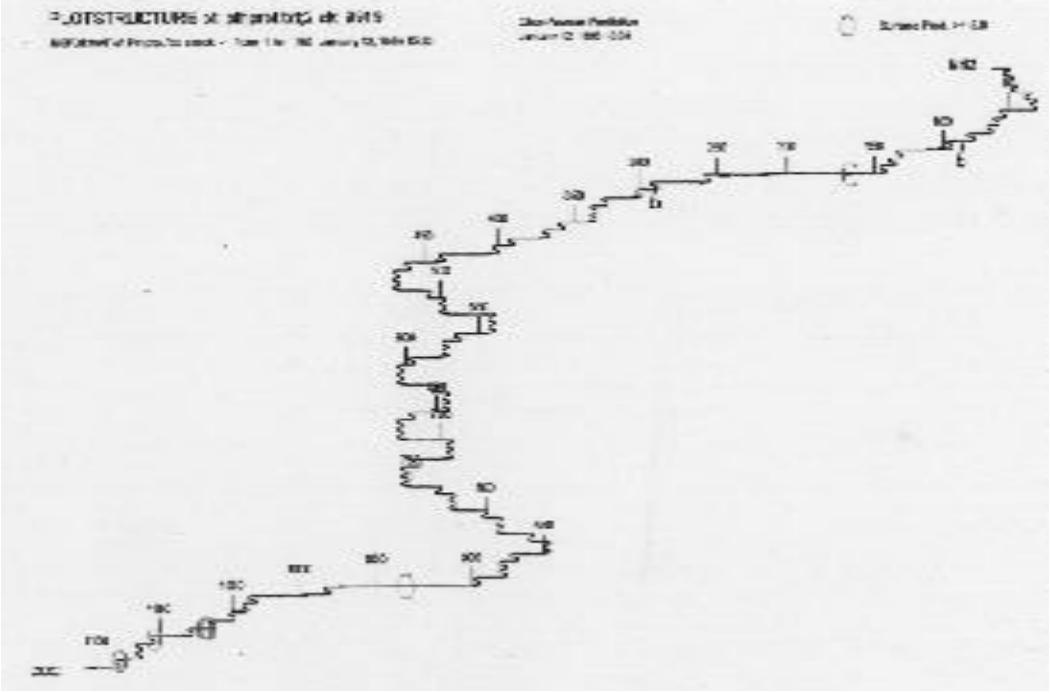


Figure 28. The predicted Surface Probability of PthA.

From all three figures, the COOH-terminal has highest chance to be exposed to the surface of the PthA protein in the natural folded state, and the COOH-terminal also has the strongest antigenic and hydrophilic characteristics. It is predicted that the COOH-terminal end of PthA full length protein plays a very important functional role in protein-protein interactions and antigenic recognition. Since the aptamers appear to interact with the COOH-terminal of PthA and block pathogenicity, the COOH terminal end is likely to be critical in the canker and HR signal transduction pathways.

In the future, the aptamer sequence could be used for permanent transformation of citrus, in attempt to engineer “resistant” or “immune” trees.

# CHAPTER 5
# SUMMARY AND CONCLUSIONS

This work was to determine if peptide aptamers could be selected that bind to PthA. Two different proteins were expressed, PthA full-length protein (constructed in pET-19b His.Tag fusion expression vector) and PthA COOH-terminal 200 amino acids of PthA fused to GST (constructed in pGEX-4T-3 expression vector). Phage display library biopanning was performed to screen 7-peptide aptamers specifically binding to full-length PthA protein and a truncated PthA consisting of the C-terminal 200 amino acids. From 11 random clones chosen from the phage population after four rounds of selection against full length PthA, the sequence encoding HPYTFLN appeared 5 times (45.5%), the sequence encoding HPHTFLN appeared twice (18.2%) and the sequence encoding YPASYMQ appeared 4 times (36.4%). From 10 random clones chosen from the phage population after three rounds of selection against the COOH-terminal 200 aa of PthA and a $4^{th}$ round of selection against full-length PthA, the sequence encoding HPYTFLN appeared twice (20.0%) and the sequence encoding YPASYMQ appeared 7 times (70.0%). ELISA tests indicated that all three aptamers, YPASYMQ, HPYTFLN or HPHTFLN, can bind to full-length PthA as well as the COOH-terminal 200 amino

acids of PthA. There were no significant differences in binding affinities among these three aptamers. Two of the aptamer sequences were cloned into pBI221. The two aptamer constructs were YPASYMQ (pGZ7.5) and HPYTFLN (pGZ7.6). Both were separately cloned into *Agrobacterium* binary vectors with and without *pthA*. They were transferred into *Agrobacterium tumefaciens* strain GV2260, and inoculated into California light-red kidney bean, sweet orange and tomato leaves. The results showed that the YPASYMQ aptamer had a strong effect to block the Hypersensitive Response (HR) normally elicited by *pthA* expressed in beans and canker elicited by *pthA* expresed in citrus. The HPYTFLN aptamer had a reduced, but significant effect. In tomato, neither aptamer reduced the HR symptom elicited by expression of *pthA* in cells. This result is in consistent with other published reports that the COOH-terminal region of other members of the *pthA* gene family are not important for elicitation of the HR in tomato. These results confirmed that the aptamers exerted their effect by binding to the COOH-terminal end of *pthA*, and indicated that aptamers might be used to control citrus canker disease.

# APPENDIX A
# MEDIA AND STRAINS USED

## Media

LB:

| | |
|---|---|
| Bacto tryptone | 10 g |
| Bacto yeast extract | 5 g |
| NaCl | 5 g |

pH was adjusted to 7.5 by adding 1N NaOH, distilled water was added up to the final volume of 1 liter, the medium was autoclaved before use.

SOBG:

| | |
|---|---|
| Tryptone | 20 g |
| Yeast extract | 5 g |
| NaCl (5 M) | 2 ml |
| KCl (1 M) | 2.5 ml |
| $MgCl_2$ (2 M) | 5 ml |
| $MgSO_4$ (1 M) | 10 ml |

Distilled water was added up to the volume of 990 ml, pH was adjusted to 7.0 by adding 1N NaOH, the medium was autoclaved and equilibrated to room temperature. At this point, 10 ml of 1M glucose (18%) was added. The final volume was 1 liter.

2X YT:

| | |
|---|---|
| Tryptone | 16 g |
| Yeast extract | 10 g |
| NaCl | 5 g |

pH was adjusted to 7.0 by adding 1N NaOH, distilled water was added up to final volume of 1 liter, the medium was autoclaved.

YEB:

| | |
|---|---|
| Beef extract | 5 g |
| Yeast extract | 1 g |
| Peptone | 5 g |
| Sucrose | 5 g |
| $MgSO_4$ | 2 mM |

pH was adjusted to 5.6 by adding 1N HCl, distilled water was added up to the final volume of 1 liter, the medium was autoclaved and equilibrated to room temperature. At this point, the following items was added:

| | |
|---|---|
| MES | 10 mM |
| Acetosyringone | 20 μM |

MMA:

| | |
|---|---|
| MS salt stock (10X) | 100 ml |
| MES | 10 mM |
| Sucrose | 20 g |
| Acetosyringone | 200 μM |

pH was adjusted to 5.6 by adding appropriate 1N HCl or 1N NaOH, distilled water was added to the final volume of 1 liter. The medium was filter sterilized.

## Strains

*E. coli* DH5α (competent cells):

*sup*E44, ΔlacU169 (ϕ80 *lac*ZΔM15*), hsd*R17, *rec*A1, *end*A1, *gyr*A96, *th*i-1, *rel*A1

*E. coli* BL21(DE3)pLysS (fusion protein expression host):

$F^-$, *ompT*, *hsdS*$_B$ ($r_B^-$, $m_B^-$), *gal*, *dcm* (DE3)pLysS, $Cm^r$ 34

*E. coli* ER2537 (M13 phage host):

$F^-$, *lacI*$^q$, *D(lac*Z*)*M15, *pro*$A^+B^+$*/fhuA2, supE, thiD(lac-proAB), D(hsdMS-mcrB)* 5($r_k^-m_k^-$McrBC$^-$)

*E.coli* HB101/pRK2073 (mating helper):

*sup*E44, *hsd*S20($r_B^-$, $m_B^-$), *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20, *xyl*-5, *mtl*-1, $Sp^r$ 50

*Agrobacterium tumefaciens* GV2260:

C58C1, $Rif^r$ 75

# APPENDIX B
# PURIFICATION OF HIS.TAG PROTEIN (FULL-LENGTH PTHA)

1. pYY50.13 Plasmid Preparation:

*E. coli* DH5α/pYY50.13 (*pthA* full-length gene in pET-19b vector) was streaked out on LB plates with ampicillin at a final concentration of 100μg/ml. A single colony was used to inoculate 2 ml of LB medium with ampicillin at a final concentration of 100 μg/ml and the inoculated culture was incubated overnight at 37°C in a rotary shaker at a speed of 220 rpm. The bacterial culture was transferred into 1.5 ml microcentrifuge tubes and centrifuged at 5,000 rpm for 5 min. The pellets were re-suspended in 200μl of Buffer P1, 200 μl of Buffer P2 was added and mixed gently, and the mixture was incubated at room temperature for 5 min. 200 μl of chilled Buffer P3 was then added and mixed immediately; the mixture was incubated on ice for 15 min. The mixture was then centrifuged at 10,000 rpm for 15 min at 4°C and the clean supernatant was transferred into a fresh 1.5 ml microcentrifuge tube. DNA was precipitated at room temperature with 0.7 volume of isopropanol for 5 min. The mixture was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was poured off. The DNA pellet was washed with 200 μl of 70% ethanol and the pellet was air-dried for 10 min. pYY50.13

DNA was re-dissolved into 30 µl of TE (pH 8.0). The quality of plasmid DNA was tested by agarose gel electrophoresis. The concentration of pYY50.13 DNA was adjusted to 1 µg/µl.

Buffer P1:

| | |
|---|---|
| Tris-HCl (pH 8.0) | 50 mM |
| EDTA | 10 mM |

Buffer P2:

| | |
|---|---|
| NaOH | 200 mM |
| SDS | 1% |

Buffer P3:

| | |
|---|---|
| Potassium acetate (pH 5.5) | 3.0M |

2. Transformation to *E. coli* BL21(DE3)pLysS:

*E. coli* BL21(DE3)pLysS competent cells were thawed on ice and of 50 µl transferred to a 1.5 ml microcentrifuge tube and mixed with 1 µl of pYY50.13 DNA on ice for 10 min without shaking. The mixture was heat shocked for 1 min at 42°C. The cells were put back on ice for 5 min. One ml of SOBG medium was added, and the mixture was incubated at 37°C in a rotary shaker at a speed of 220 rpm for 45 min. 150 µl of transformed culture was spread on LB agar plates with carbenicillin at a final concentration of 50µg/ml and chloramphenicol at a final concentration of 34 µg/ml, and the plates were inverted and incubated at 37°C overnight.

3. Expression of *E. coli* pYY50.13/BL21(DE3)pLysS (PthA full-length protein):

A single colony from the above transformants was inoculated in 2 ml of LB broth with carbenicillin at a final concentration of 50 μg/ml and chloramphenicol at a final concentration of 34 μg/ml as a starter culture and incubated in a rotary shaker at a speed of 220 rpm at 37°C overnight. 500 μl of the overnight culture was diluted 1:100 into 50 ml of LB with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol in a 250 ml flask, and continuously incubated in a rotary shaker at a speed of 220 rpm for 3 to 5 hours at 37°C till $OD_{600}$=0.5. For incubation, 50μl of 100mM IPTG was added to 50 ml of early-log-phase bacterial culture (the final concentration of IPTG was 0.1mM) and the culture was incubated in a rotary shaker at a speed of 220 rpm at 28°C for 3 hr. The bacterial culture was centrifuged at 7,000 rpm at 4°C for 10 min. The pellet was washed once with 1X Binding Buffer. The cells were re-suspended in 5 ml of 1X Binding Buffer. The suspensions were kept on ice.

1X Binding Buffer:

| | |
|---|---|
| Imidazole | 5 mM |
| NaCl | 0.5 M |
| Tris-HCl (pH 7.9) | 20 mM |

4. His.Bind Resin Preparation:

His.Bind Resin (Novagen) was gently mixed by inversion until the slurry was completely suspended. The slurry was transferred into a 10 ml column (1cm in diameter). The storage buffer (with 20% ethanol) was completely removed, and the

remaining resin occupied about 1 ml of settled bed volume. The column was washed, charged and equilibrated with 3 volumes of sterile deionized water, 5 volumes of 1X Charge Buffer and 3 volumes of 1X Binding Buffer, respectively.

1X Charge Buffer:

| | |
|---|---|
| $NiSO_4$ | 50 mM |

5. PthA Cell Extract Preparation:

0.1% NP-40 and 1mM PMSF was added to the chilled cell suspension in 1X Binding Buffer. The mixture was kept on ice for 10 min. The cell mixture was sonicated with SONICATOR (Model W-225) (% duty cycle=50, out put control=6, pulsed cycle) for 60 seconds in a ice bath until the sonicated solution was no longer viscous. The sonicated mixture was kept on ice. The lysate was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was transferred to a fresh tube and filtered through a 0.45 micron membrane. About 5 ml of prepared crude extract was collected and kept on ice until the next step.

6. His.Tag Column Chromatography to Purify PthA:

The 1X Binding Buffer was allowed to drain to the top of the column bed. 5 ml of prepared crude extract was loaded on the top of the column and the column was adjusted to a flow rate of about 10 column volumes per hour. When all cell extract had passed through, the column was washed with 10 volumes of 1X Binding Buffer and 6 volumes of 1X Wash Buffer. The bound PthA was finally eluted with 6 volumes of 1X Elute Buffer. The PthA eluate (about 5ml) was collected in a fresh tube which was kept

on ice. The column was cleaned with 5 volumes of 1X Strip Buffer. Finally, the column was washed with deionized water until equilibrium was achieved.

1X Wash Buffer:

| | |
|---|---|
| Imidazole | 60mM |
| NaCl | 0.5M |
| Tris-HCl (pH 7.9) | 20mM |

1X Elute Buffer:

| | |
|---|---|
| Imidazole | 1M |
| NaCl | 0.5M |
| Tris-HCl (pH 7.9) | 20mM |

1X Strip Buffer:

| | |
|---|---|
| EDTA | 100mM |
| NaCl | 0.5M |
| Tris-HCl (pH 7.9) | 20mM |

7. Dialysis of PthA:

The PthA column eluate (about 5 ml) was transferred into SPECTRAPOR membrane tubing which was dialyzed against 50 mM $NH_4HCO_3$ at 4°C with gentle agitation overnight. The dialysis solution was changed in every 2 hrs.

8. Lyophilization of PthA:

The dialyzed PthA was pipetted out of the dialysis bag and put into Falcon 14 ml centrifuge tubes. The protein sample was frozen at -70°C overnight. The dialyzed PthA was freeze-dried in a SENTRY Freeze Dryer overnight.

9. SDS-Polyacrylamide Gel Electrophoresis of PthA:

The freeze dried PthA was re-suspended in 200 µl of 1X Treatment Buffer, The mixture was boiled for 10 min and then centrifuged at 10,000 rpm for 10 min. Only the upper 80% supernatant of treated samples was pipetted out for electrophoresis loading.

1X Treatment Buffer:

| | |
|---|---|
| Tris-HCl (pH 6.8) | 0.125 M |
| SDS | 4% |
| Glycerol | 20% |
| DTT | 0.2 M |
| Bromophenol Blue (pH 6.8) | 0.02% |

Protein samples were separated by SDS-PAGE, using an 8% separating gel and 4% stacking gel.

8% Separating gel:

| | |
|---|---|
| distilled water | 16.37 ml |
| 1.5 M Tris-HCl (pH 8.8) | 8.75 ml |
| 30% Acrylamide/Bis | 9.3 ml |
| 10% SDS | 350 µl |

| | |
|---|---|
| 10% Ammonium persulfate | 210 µl |
| TEMED | 21 µl |

4% Stacking gel:

| | |
|---|---|
| distilled water | 8.54 ml |
| 0.5 M Tris-HCl (pH 6.8) | 3.5 ml |
| 30% Acrylamide/Bis | 1.82 ml |
| 10% SDS | 140 µl |
| 10% Ammonium persulfate | 75 µl |
| TEMED | 20 µl |

30% Acyrlamide/Bis (30%T, 2.67%C) stock:

| | |
|---|---|
| Acrylamide | 29.2 g |
| N, N'-bis-methylene-acrylamide | 0.8 g |

(Distilled water was added up to 100 ml. The solution was stored at 4°C in dark.)

SDS-PAGE was run at 100 V for 5 hr. After gel electrophoresis, the gel slice was soaked into 200 ml of ice-cold 0.2 M KCl. The gel slice was kept on ice for 2 min. Proteins appeared as white pale colored bands on the gel slice. A razor blade was used to cut the 130 kD protein band out and the recovered gel slice was chopped into small pieces.

10. Gel Elution of PthA:

The recovered gel slices were loaded into Model 422 Electro-Eluter (Bio-Rad) assembly. The gel slices were washed with distilled water for 5 min to eliminate excess KCl. PthA was electroeluted at 10 mA per tube for 8 hours. The Protein Elution Buffer without SDS was changed at this point and electrophoresis was continued for an additional 30 min to remove SDS in the protein sample.

Protein Elution Buffer:

| | |
|---|---|
| Tris base | 25 mM |
| Glycine | 192 mM |
| SDS | 0.1% |

The PthA eluate was collected in a volume of 700 µl, which was transferred into SPECTRAPOR membrane tubing and dialyzed against 50mM $NH_4HCO_3$ at 4°C overnight with gentle agitation. The dialyzed PthA electro gel eluate was freeze-dried overnight.

11. PthA Quantification by UV Spectrophotometry:

PthA was re-dissolved in 100 µl of 0.1M $NaHCO_3$ (pH 8.6), and 10 µl was diluted 1:30 in 300 µl of 0.1M $NaHCO_3$ (pH 8.6). The optical density was measured in a Gilford spectrophotometer. The $OD_{280}$ was 0.3363, the $OD_{260}$ was 0.3286, the $A_{280}/A_{260}$ ratio was 1.023. The concentration of electro gel purified PthA was caculated by $A_{280}$ X Correction Factor = 0.3363 X 0.81 = 0.27 mg/ml. The original concentration of PthA in 0.1M $NaHCO_3$ (pH 8.6) was 8.1 mg/ml. PthA were stored at -20°C.

# APPENDIX C

## PURIFICATION OF GST FUSION PROTEIN (PTHA C-TERMINAL 200AA TRUNCATED PROTEIN)

1. pGNLS-3-2 Plasmid Preparation:

*E. coli* DH5α/pGNLS-3-2 (*pthA* C-terminal 600bp truncated gene fused with GST in pGEX-4T-3 vector) plasmid DNA was prepared as described in Appendix B1.

2. Transformation to *E. coli* BL21(DE3)pLysS:

*E. coli* BL21(DE3)pLysS competent cells were prepared as described in Appendix B2.

3. Expression of *E. coli* pGNLS-3-2/BL21(DE3)pLysS (PthA C-terminal 200aa Truncated Protein):

*E. coli* pGNLS-3-2/BL21(DE3)pLysS cells were induced as described in Appendix B3, except that the culture medium was 2X YT medium, the cells were washed and resuspended in 1X PBS.

1X PBS (pH 7.3):

| | |
|---|---|
| NaCl | 140 mM |
| KCl | 2.7 mM |

| | |
|---|---|
| $Na_2HPO_4$ | 10 mM |
| $KH_2PO_4$ | 1.8 mM |

4. Preparation of Glutathione Sepharose 4B:

The bottle of Glutathione Sepharose 4B (Pharmacia Biotech) was gently shaken to resuspend the matrix. The matrix was washed with 1X PBS. A 50% Glutathione Sepharose 4B slurry was prepared in a bed volume of 50 µl. A micropipette was used to remove 100 µl of slurry and the slurry was transferred to a 1.5 ml microcentrifuge tube. The matrix was sedimented by centrifugation at 500 x g for 5 min. The supernatant was carefully removed and decanted. The matrix was washed again with 700 µl of cold 1X PBS, inverted to mix, and centrifuged at 500 x g for 5 min. Again, the supernatant was discarded. The matrix was kept at 4°C ready for use.

5. GST Fusion Cell Extract Preparation:

The truncated PthA::GST fusion protein was prepared as described in Appendix B5.

6. Glutathione Sepharose 4B Batch Purification of GST Fusion:

One ml of the prepared cell extract was added to the Glutathione Sepharose 4B affinity matrix in a 1.5 ml microcentrifuge tube; the tube was slowly inverted to mix with gentle agitation at 4°C for 30 min. The slurry was centrifuged at 500 x g for 5 min and the supernatant was carefully removed. One ml of cold 1X PBS was added and inverted to mix with gentle agitation at 4°C for 10 min. The mixture was centrifuged at 500 x g for 5 min and the supernatant was carefully removed. The washing steps was repeated

twice. One ml of Glutathione Elution Buffer was added, the tube was inverted to mix with gentle agitation at 4°C for 30 min. The slurry was centrifuged at 500 x g for 10 min. The supernatant was kept on ice as "glutathione protein elute". The rest of Glutathione Sepharose 4B matrix should be washed by 1X PBS at least three times to clean up.

Glutathione Elution Buffer:

| | |
|---|---|
| Reduced glutathione | 10 mM |
| Tris-HCl (pH 8.0) | 50 mM |

7. Dialysis of GST Fusion Protein:

The total Glutathione eluate (about 5 ml) was transferred into SPECTRAPOR membrane tubing and dialyzed against 50mM $NH_4HCO_3$ at 4°C overnight. Fresh solution was changed every two hours. The solution was agitated at very low speed.

8. Lyophilization of GST Fusion:

The dialyzed Glutathione eluate was freeze-dried as described in Appendix B8.

9. SDS-PAGE of GST Fusion Protein:

The freeze-dried Glutathione protein sample was electrophoresed as described in Appendix B9. The 55 kD protein band was recovered.

10. Gel Elution of GST Fusion Protein:

The GST fusion protein was electro-eluted as described in Appendix B10.

11. Protein Quantification by UV Spectrophotometry:

The concentration of PthA C-terminal 200aa truncated protein in 0.1M $NaHCO_3$

(pH 8.6) was 10.0 mg/ml, determined as described in Appendix B11. The GST fusion protein sample was stored at -20°C.

# APPENDIX D

# PHAGE DISPLAY PEPTIDE LIBRARY BIOPANNING – SCREENING OF APTAMERS

## Phage Display Biopanning

1. A solution of 100 μg/ml of the target in 0.1M $NaHCO_3$ (pH 8.6) was prepared. There were two targets: PthA full-length protein (His.Tag) and the PthA C-terminal 200aa truncated protein (GST fusion protein). Also, a solution of 100 μg/ml of Glutathione S-transferase (GST) was prepared as a pre-absorb target in 0.1 M $NaHCO_3$ (pH 8.6).

2. 150 μl of each target was added to two separate microtiter wells. In a separate well, 150 μl of GST was added. The microtiter plate was swirled repeatedly until the surface was completely wet. The plate was incubated at 4°C overnight with gentle agitation within a humidified container.

3. *E. coli* ER2537 stock culture was inoculated into 10 ml of LB medium, and incubated with vigorous shaking at a speed of 220 rpm at 37°C overnight. The overnight culture was 1:100 diluted into 100 ml LB, and continuously incubated at 37°C in a rotary shaker at a speed of 220 rpm for 3 hr until the $OD_{600}$ reached 0.5.

4. The coating solution from each well was poured off and the plate was firmly slapped face-down onto a clean paper towel to remove residual solution. Each plate or well was completely filled with Blocking Buffer and incubated at least 1 hour at 4°C with gentle agitation.

Blocking Buffer:

| | |
|---|---|
| $NaHCO_3$ (pH 8.6) | 0.1 M |
| BSA | 5 mg/ml |
| $NaN_3$ | 0.02% |

5. The Blocking Buffer was discarded by shaking. The well was washed 6 times with TBST at room temperature.

TBST:

| | |
|---|---|
| Tris-HCl (pH 7.5) | 50 mM |
| NaCl | 150 mM |
| Tween-20 | 0.1% (v/v) |

6. $2 \times 10^{11}$ M13 phage from the original library (New England BioLabs, Ph.D.-7™ Phage Display Heptapeptide Library, $2 \times 10^{13}$ pfu/ml) was diluted by 1 ml of TBST. The diluted phage were pipetted onto coated plates and rocked gently for 60 min at room temperature. For PthA full-length protein (His.Tag) screening, the diluted phage was directly added. For the GST fusion protein screening, the diluted phage was first added to the GST coating well and incubated for 60 min, then the pre-absorbed phage was

transferred to the GST fusion protein coating well and incubated for another 60 min at room temperature. Such a pre-absorbing step was proved to be very efficient to eliminate GST non-specific binding background.

7. The non-binding phage was discarded and the plate was washed 10 times with 1X TBST.

8. The bound phage were eluted with 100 µl of free target solutions, i.e., 100 µg/ml of PthA full-length protein (His.Tag) in TBS and 100 µg/ml of GST fusion protein in TBS, respectively. The plates were rocked gently for 60 min at room temperature. The eluate was transferred to a 1.5 ml microcentrifuge tube.

TBS:

| | |
|---|---|
| Tris-HCl (pH 7.5) | 50 mM |
| NaCl | 150 mM |

9. One µl of elute phage was titered based on the following steps. The remaining phage elute was stored at 4°C.

10. The elute phage was amplified till the titer up to $2 \times 10^{13}$ pfu/ml.

11. The biopanning steps were repeated twice. The only difference was changing the Tween-20 to 0.5% in washing steps after second round phage display biopanning. For GST fusion protein, each round of selection was pre-absorbed by GST.

## Phage Amplification

1. 10 ml of LB medium was inoculated with a single colony of *E. coli* ER2537 and was shaken vigorously in a rotary shaker at a speed of 220 rpm at 37°C overnight. The overnight starting culture was diluted 1:100 into 100 ml of LB medium. The dilute bacterial culture was incubated at 37°C for 3 hr until the $OD_{600}$ reached 0.5.

2. One microliter of phage eluate was added into 20 ml of early-log-phase *E. coli* ER2537 culture, transferred to a 250 ml flask, and incubated at 37°C in a rotary shaker at a speed of 220 rpm for 4.5 hr.

3. The culture was centrifuged at 10,000 rpm for 15 min at 4°C. The upper 80% supernatant was transferred to a fresh tube and re-centrifuged briefly.

4. The upper 80% of the supernatant was pipetted out to a fresh tube and 1/6 volume of PEG/NaCl was added. The M13 phage was precipitated at 4°C overnight.

PEG/NaCl:

| | |
|---|---|
| Polyethylene glycol-8000 | 20% (w/v) |
| NaCl | 2.5 M |

5. The precipitation mixture was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was discarded. The residual supernatant was carefully removed by a pipette.

6. The pellet was re-suspended in 1 ml of TBS. The suspension was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 10,000 rpm for 5 min at 4°C to remove the residual cells.

7. The supernatant was transferred to a fresh tube and re-precipitated by adding 1/6 volume of PEG/NaCl. The precipitation mixture was incubated on ice for 60 min then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the residual supernatant was carefully removed by a micropipette.

8. The pellet was resuspended in 200 µl of TBS with 0.02% $NaN_3$. The suspension was centrifuged at 10,000 rpm for 5 min to pellet any remaining insoluble matter. The supernatant was transferred to a fresh tube. This was the amplified phage.

9. The amplified phage was titered according to the next step. All the eluate were stored at 4°C.

## Phage Titer

1. Ten ml of LB medium was inoculated with a single colony of *E. coli* ER2537 and incubated in a rotary shaker at a speed of 220 rpm at 37°C overnight. The overnight culture was 1:100 diluted into 100 ml LB medium and continuously incubated at 37°C for 3 hr to reach $OD_{600}$=0.5.

2. Agarose Top Agar was melted in a microwave oven and 3 ml of melted top agar was dispensed into a 15ml Falcon centrifuge tubes (one tube for one phage dilution). All the tubes were equilibrated at 45°C water bath ready for use.

Agarose Top Agar:

| | |
|---|---|
| Bacto-tryptone | 10 g |

| | |
|---|---|
| Yeast extract | 5 g |
| NaCl | 5 g |
| $MgCl_2.6H_2O$ | 1 g |
| Agarose | 7 g |

pH was adjusted to 7.5, $H_2O$ was added up to volume of 1 liter, the medium was autoclaved and stored in room temperature.

3. LB agar plates were pre-warmed at 37°C (one plate per phage dilution).

4. Ten-fold serial dilutions of M13 phage were prepared in LB medium. The dilution range was from $10^1$ to $10^{12}$.

5. 200 µl of mid-log *E. coli* ER2537 culture was transferred into a 1.5 ml microcentrifuge tube (one tube for one phage dilution).

6. 10 µl of each phage dilution was pipetted to each microcentrifuge tube with bacteria, mixed quickly and incubated at room temperature for 1 min (The infection time was very critical).

7. The infected cells were transferred to the 45°C pre-warmed Agarose Top agar tube, mixed well, and were immediately poured onto a 37°C pre-warmed LB agar plate. Top agar was spread evenly by tilting plate for several times.

8. The plate was cooled at room temperature for 5 min, inverted and incubated at 37°C no longer than 18 hours. Longer incubation may cause deletion of the phage sequence.

9. The plates were inspected and the phage plaques was counted. A good range of

dilution was the plate having about 100 plaques. The dilution factor was multiplied to get phage titer in plaque forming units (pfu) per ml.

## Characterization of Binding Clones

1. 10 ml of LB medium was inoculated with a single colony of *E. coli* ER2537 and the inoculated culture was incubated in a rotary shaker at a speed of 220 rpm at 37°C overnight. The overnight starting culture was diluted 1:100 into 100 ml of fresh LB medium, and continuously incubated at 37°C for 3 hr to reach $OD_{600}$=0.5.

2. One milliliter of early-log-phase bacterial culture was transferred into 15 ml test tubes (one for each clone to be characterized).

3. The phage titer plate with about 100 plaques was chosen. A sterile wooden stick was used to stab each plaque and transfer to the bacterial culture tube.

4. The test tube with bacteria and phage mixture was incubated at 37°C in a rotary shaker at a speed of 220 rpm for 4.5 hr.

5. The culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged at a speed of 10,000 rpm for 1 min. The upper 80% of the supernatant was transferred to a fresh tube and re-centrifuged for an additional 1 min. The upper 80% of the supernatant was transferred to another fresh tube.

6. 300 µl of the upper 80% of the supernatant was diluted 1:1 with 100% glycerol and stored at -20°C as the phage stock. It could be re-amplified as necessary.

7. The rest of 500 µl of upper 80% supernatant was transferred to a fresh 1.5 ml tube.

8. 200 µl of PEG/NaCl was added, the tube was inverted several times to mix well, and incubated in room temperature for 10 min.

9. The mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was discarded.

10. The pellet was re-suspended in 100 µl of Iodide Buffer and 250 µl of 95% ethanol was added later. The mixture was incubated at room temperature for 10 min.

Iodide Buffer:

| | |
|---|---|
| Tris-HCl (pH 8.0) | 10 mM |
| EDTA | 1 mM |
| NaI | 4 M |

11. The mixture was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discanted. The pellet was carefully washed by 70% ethanol and dried by a vacuum.

12. The pellet was re-suspended in 30 µl of TE (pH 8.0).

13. The single-stranded M13 phage DNA was tested by 0.7% agarose gel electrophoresis.

14. 10 µl of each sample was enough for DNA sequencing. The DNA sequencing primer was -28gIII (5'-GTATGGGATTTTGCTAAACAAC-3') which was synthesized by University of Florida ICBR DNA Synthesis Core Facility.

## APPENDIX E
## *IN VITRO* BINDING AFFINITY OF APTAMERS - ELISA

1. Host strain *E coli* ER2537 was streaked out in a LB agar plate.

2. A single colony was inoculated to 2 ml of LB medium as starting culture. The culture tube was incubated in a rotary shaker at a speed of 220 rpm at 37°C overnight.

3. The overnight starting culture was diluted 1:100 into 20 ml of LB medium in a 250 ml flask, incubated in a rotary shaker with a shaking speed of 220 rpm at 37°C for about 3 hr, until the $OD_{600}$ reached 0.5 (early-log-phase).

4. 1 μl of M13 amplified phage stock (phage upper 80% supernatant 1:1 mixed with 100% glycerol, stored at -20°C) was transferred to the 20ml of early-log-stage *E. coli* ER2537 culture, and the inoculated culture was incubated in a rotary shaker with a shaking speed of 220 rpm at 37°C for 4.5 hr.

5. The culture was transferred to a Falcon centrifuge tube and centrifuged at 10,000 rpm at 4°C for 10 min. The upper 80% of the supernatant was transferred to a fresh tube and re-centrifuged at 10,000 rpm at 4°C for 5 min.

6. The upper 80% supernatant was pipetted out to a fresh tube and 1/6 volume of PEG/NaCl was added to the mixture. The M13 phage was precipitated overnight at 4°C.

7. The precipitation mixture was centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant was discarded. The residual supernatant was carefully removed by a micro pipette.

8. The pellet was re-suspended in 1 ml of TBS.

9. The phage suspension was transferred into a 1.5 microcentrifuge tube, centrifuged at 10,000 rpm at 4°C for 5 min to remove the residual cells.

10. The upper supernatant was transferred to a fresh 1.5 ml tube. The M13 phage was re-precipitated by adding 1/6 volume of PEG/NaCl at 4°C overnight.

11. The overnight precipitation mixture was centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant was discarded. The residual supernatant was carefully removed by a micro pipette.

12. The pellet was re-suspended 50 µl of TBS.

13. The phage suspension was titered. The phage titer should be more than $10^{13}$ pfu/ml.

14. As a phage control, 1 µl of M13 phage stock was removed from the original library (New England BioLabs, Ph.D.-7$^{TM}$ Phage Display Heptapeptide Library, 2 x $10^{13}$ pfu/ml) and amplified a titer of $10^{13}$ pfu/ml.

15. 0.5 ml microcentrifuge tubes were coated with 150 µl of 100 µg/ml of target in 0.1 M $NaHCO_3$ (pH 8.6). There were two targets: PthA full-length protein (His.Tag) and PthA C-terminal 200 aa truncated protein (GST fusion protein). As a target control, one set of tubes was coated with 150 µl of 100 µg/ml of BSA in 0.1 M $NaHCO_3$ (pH 8.6).

16. The excess target solution was shaken out. The coated tubes were centrifuged briefly, the residual solution was carefully removed by a micro pipette. 200 µl of Blocking Buffer was added to each tube. All tubes were incubated at 4°C for 2 hr.

Blocking Buffer:

| | |
|---|---|
| $NaHCO_3$ (pH 8.6) | 0.1 M |
| BSA | 5 mg/ml |
| $NaN_3$ | 0.02% |

17. In one set of empty tubes, only 200 µl of Blocking Buffer was added and incubated at 4°C for 2 hr.

18. The 10-fold serial dilution of M13 phage was prepared in 1 X TBST. The dilution range was from $10^{12}$ pfu/ml to $10^{7}$ pfu/ml.

1 X TBST:

| | |
|---|---|
| Tris-HCl (pH 7.5) | 50 mM |
| NaCl | 150 mM |
| Tween-20 | 0.1% (v/v) |

19. The Blocking Buffer of each tube was shaken out. All tubes were washed 6 times with 1X TBST (Tween-20 0.5% v/v).

20. 200 µl of serial dilution of M13 phage was incubated in the only-blocked tubes without any target as pre-absorbing for 1 hr at room temperature.

21. The pre-absorbed serial dilution phage was transferred into the blocked tube with preferred target by a micropipette. The infection mixture of phage and target was incubated at room temperature for 1 hr.

22. Each tube was washed 6 times with 1 X TBS/Tween-20 (0.5% v/v).

23. The HRP/Anti-M13 Monoclonal Conjugate (Pharmacia) was diluted 1:5000 in Blocking Buffer. 200 µl of diluted Monoclonal conjugate was added to each tube and incubated at room temperature for 1 hr.

24. Each tube was washed 6 times with 1 X TBS/Tween-20 (0.5% v/v).

25. Sigma FAST OPD (Sigma, O-Phenylenediamine Dihydrochloride) substrate was prepared according to the instruction manual. One tablet of OPD and one tablet of Urea Hydrogen Peroxide were dissolved in 20 ml $dH_2O$. The cylinder was wrapped by aluminum foil to avoid light. The solution must be used within one hour and stored in dark at room temperature.

26. 200 µl of fresh Sigma FAST OPD substrate was added to each tube. The reaction tube was incubated in dark at room temperature for 30 min.

27. 50 µl of 3M HCl was added to each tube to stop reaction.

28. 150 µl of reaction mixture in each tube was transferred to a 96-well ELISA plate (Corning) only for ELISA microplate reading.

29. Microplate Autoreader EL 309 (Bio-Tek Instruments) was set up to read the plate at 492 nm.

30. The data were recorded and analyzed using the Sigma Plot software package.

# APPENDIX F
# PCR-UDG CLONING OF APTAMERS

1. PCR Template Preparation:

*E. coli* DH5α/pYD12.9F clone (*pthA* gene fused with GUS in pBI221 vector and with a synthetic ribosome binding site (RBS) )was streaked out from stock culture (stored at -70°C bio-freezer) on a LB agar plate with ampicillin at a final concentration of 100 μg/ml. A single colony was picked up to inoculate 2 ml LB medium with ampicillin at a final concentration of 100 μg/ml. The inoculated medium was incubated at 37°C overnight in a rotary shaker in a speed of 220 rpm. The bacterial culture was transferred to 1.5ml microcentrifuge tubes and centrifuged at 5,000 rpm for 5 min. The cell pellet was collected and re-suspended in 200 μl of Buffer P1. 200 μl of Buffer P2 was added, and mixed gently. The mixture was incubated at room temperature for 5 min. 200 μl of chilled Buffer P3 was added and mixed immediately. The mixture was incubated on ice for 15 min and then centrifuged at 10,000 rpm for 15 min at 4°C. The clean supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. 0.7 volumes of isopropanol was added and the DNA was precipitated at room temperature for 5 min. The precipitation

mixture was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was discarded. The pellet was washed with 200 µl of 70% ethanol. The access ethanol was carefully removed by a micropipette and the pellet was briefly air-dried for 10 min. pYD12.9F plasmid DNA was re-dissolved into 30 µl of TE (pH 8.0). The DNA concentration and quality was tested by 0.7% agarose gel electrophoresis. The estimated DNA concentration was 1 µg/µl.

pYD12.9F DNA was digested by *Bam*HI as follows: 20 µl of pYD12.9F DNA , 4 µl of 10X REact Buffer 3, 1 µl of *Bam*HI, 5 µl of RNase (200 µg/ml) and 10 µl of $dH_2O$. The digestion mixture was incubated at 37°C for 1 hr. 500 µl of TE (pH 8.0) was added into the digestion mixture and an equal volume of Phenol/Chloroform/IAA (25:24:1) was added to remove protein. The DNA was precipitated by adding 1 ml of 95% ethanol and 50 µl of 3M sodium acetate (pH 5.3) at -20°C overnight. The mixture was centrifuged at 10,000 rpm for 15 min at 4°C, the pellet was washed by 70% ethanol, briefly air-dried for 10 min at room temperature, and re-dissolved in 20 µl of TE (pH 8.0). DNA concentration was estimated by comparison with DNAs of known concentration using agarose gel electrophoresis. The concentration of pYD12.9F/*Bam*HI was 1 µg/µl.

1 µl pYD12.F/*Bam*HI (1 µg/µl) was diluted in 199 µl TE (pH 8.0) as a PCR template (1:200 v/v dilution).

2. PCR Primer Reconstitution:

Primer GZ1 (GIBCO BRL): 43.6 nmoles was added to 100 µl TE (pH 8.0); the

reconstituted concentration of primer GZ1 was 436 μM. 1 μl of GZ1 reconstitute was diluted in 99 μl TE (pH 8.0), the final working concentration of primer GZ1 was 4.36 μM (Tm=58°C).

Primer GZ2 (GIBCO BRL): 43.2 nmoles was added to 100 μl TE (pH 8.0); the reconstituted concentration of primer GZ2 was 432 μM. 1 μl of GZ2 reconstitute was diluted in 99 μl TE (pH 8.0), the final working concentration of primer GZ2 was 4.32 μM (Tm=54°C).

Primer GZ3 (GIBCO BRL): 47.3 nmoles was added to 100 μl TE (pH 8.0); the reconstituted concentration of primer GZ3 was 473 μM. 1 μl of GZ3 reconstitute was diluted in 99 μl TE (pH 8.0), the final working concentration of primer GZ3 was 4.73 μM (Tm=58°C).

Primer GZ4 (GIBCO BRL): 48.2 nmoles was added to 100 μl TE (pH 8.0); the reconstituted concentration of primer GZ4 was 482 μM. 1 μl of GZ4 reconstitute was diluted in 99 μl TE (pH 8.0), the final working concentration of primer GZ4 was 4.82 μM (Tm=54°C).

All reconstituted and diluted primers were stored at -20°C.

3. PCR Reactions:

① Reaction I

| | |
|---|---|
| Template (pYD12.F/*Bam*HI, 1:200 v/v dilution) | 2μl |
| 10X PCR Buffer | 15μl |

| | |
|---|---|
| 50 mM $MgCl_2$ | 4.5µl |
| 2.5 mM dNTP | 15µl |
| GZ1 working primer (4.36 µM) | 15µl |
| GZ2 working primer (4.32 µM) | 15µl |
| $dH_2O$ | 82.75µl |

② Reaction II

| | |
|---|---|
| Template (pYD12.F/*Bam*HI, 1:200 v/v dilution) | 2µl |
| 10X PCR Buffer | 15µl |
| 50 mM $MgCl_2$ | 4.5µl |
| 2.5 mM dNTP | 15µl |
| GZ3 working primer (4.73 µM) | 15µl |
| GZ4 working primer (4.82 µM) | 15µl |
| $dH_2O$ | 82.75µl |

The reaction mixture was mixed well in a 0.5 ml microcentrifuge tube. A negative control (without template) was set up. 100 µl of mineral oil was added on top of each reaction mixture. After preheating the reaction mixture at 95°C for 4 min, 0.75 µl of Taq Polymerase (5 U/µl) was added (The total reaction volume should be 150 µl at this point), then the Cycle 19 of Program 09 was continued. The detailed PCR programs were as follows:

PCR Program 09:

Cycle 1: 95°C, 4 min

Cycle 19: 94°C, 1 min

56°C, 1 min

72°C, 5 min

Cycle 20: 92°C, 1 min

55°C, 1 min

72°C, 5 min

Cycle 21: 92°C, 1 min

54°C, 1 min

72°C, 5 min

Cycle 22: 92°C, 1 min

53°C, 1 min

72°C, 5 min

Cycle 23: 92°C, 1 min

52°C, 1 min

72°C, 5 min

Cycle 24: 92°C, 1 min

51°C, 1 min

72°C, 5 min

Cycle 25: 92°C, 1 min

50°C, 1 min

72°C, 5 min

Cycle 26: 94°C, 1 min

65°C, 1 min

72°C, 5 min

Cycle 2: 72°C, 5 min

Cycle 3: 25°C, 3 hr

After PCR, the reaction mixture was stored at -20°C overnight. The oil was carefully removed by a micropipette. The PCR product was tested by 0.7% agarose gel electrophoresis.

4. UDG Treatment of PCR Product:

| | |
|---|---|
| PCR product | 5 µl |
| 10X PCR Buffer | 2 µl |
| Uracil DNA Glycosylase (1 U/µl) | 1 µl |
| $dH_2O$ | 12 µl |

The mixture was incubated at 37°C for 30 min. Uracil DNA Glycosylase was denatured at 65°C for 10 min. The PCR-UDG product was stored at 4°C.

5. Transformation of PCR-UDG Product:

50 µl of thawed *E. coli* DH5α competent cells were transferred to a 1.5 ml microcentrifuge tube and mixed with 5 µl of PCR-UDG product. The mixture was incubated on ice for 10 min. The cells were heat shocked for 1 min at 42 °C then put back on ice for 5 min. One ml of SOBG medium was added and the mixture was incubated at 37°C in a rotary shaker at a speed of 220 rpm for 45 min. 150 µl of transformed culture was spread on LB agar plate with ampicillin at a final concentration of 100 µg/ml. The plate was inverted and incubated at 37°C overnight.

6. Identification of the PCR-UDG Product:

Single colonies of the above transformants were inoculated into 2 ml of LB medium with ampicillin at a final concentration of 100 µg/ml. The inoculated medium was incubated at 37°C overnight with vigorous shaking. Plasmid DNA was prepared and digested by *Xba*I/*Bam*HI and *Xba*I/*Sma*I, respectively. The *Xba*I/*Bam*HI reaction mixture was set up as follows: 2 µl of DNA, 2 µl of 10X REact Buffer 6, 1 µl of *Xba*I, 1 µl of *Bam*HI, 1 µl of RNase (200 µg/ml) and 13 µl of $dH_2O$. The *Xba*I/*Sma*I reaction mixture was set up as follows: 2 µl of DNA, 2 µl of 10X REact Buffer 9, 1 µl of *Xba*I, 1 µl of *Sma*I, 1 µl of RNase (200 µg/ml) and 13 µl of $dH_2O$. The digestion mixture was incubated at 37°C for 1 hr. The digestion products were examed by 2.0% agarose gel electrophoresis.

7. Polyacrylamide Gel Electrophoresis of Small DNA Fragments:

DNA from interesting clones was extracted, digested and run on 15.0% polyacrylamide gels to separate DNA fragments ranging in size from 25 bp up to 150 bp. A BIO-RAD Mini-PROTEAN II Electrophoresis Cell was set up for polyacrylamide gels as follows:15.0% acrylamide,1X TBE, 0.07% 10% ammonium persulfate. The acrylamide gel mixture was de-gassed and mixed well. 3.5 µl of TEMED was added to each 10 ml of acrylamide solution. 10 µl of digested DNA sample was loaded in each lane. The electrophoresis molecular weight marker was Promega 100 bp DNA Ladder.

30% Acrylamide:

| | |
|---|---|
| Acrylamide | 29 g |
| N,N'-methylenebisacrylamide | 1 g |
| $dH_2O$ | 100 ml |

5X TBE:

| | |
|---|---|
| Tris base | 54 g |
| Boric acid | 27.5 g |
| 0.5 M EDTA (pH 8.0) | 20 ml |

$dH_2O$ was added up to the final volume of 1 liter.

8. Glycerol Stock Culture:

1.52 ml of overnight culture was mixed with 0.48 ml of 50% glycerol and

transferred into 2 ml screw-cap free standing tube. The glycerol stock culture was stored at -70°C.

9. GUS Activity Assay:

One ml of overnight culture was transferred to a 1.5 ml micro-centrifuge tube and centrifuged at 5,000 rpm at 4°C for 5 min. The pellet was re-suspended in 570 µl of $dH_2O$. 330 µl of 0.3 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) was added. The mixture was incubated at room temperature overnight. Very strong blue color developed in the positive clones after 12 hrs compared to no color change in the *E. coli* DH5α control.

10. Clones Purification for DNA Sequencing:

The DNA from interesting clones was purified using a QIAGEN Plasmid Midi Kit. DNA quality was tested by 0.7% agarose gel electrophoresis. One µl of plasmid DNA was compared to 1 µg of λDNA/*Hind*III fragments and 1 µg of 1Kb DNA Ladder. The concentration of plasmid DNA sample for sequencing was adjusted to 1 µg/µl. The DNA sequencing primer was DG39R (5'- CAT AAG GGA CTG ACC A -3'), which was synthesized by University of Florida ICBR DNA Synthesis Core Facility.

Two interesting clones were identified: pGZ7.5 (YPASYMQ in pBI221) and pGZ7.6 (HPYTFLN in pBI221). Aptamers sequences were engineered to fuse with a GUS reporter gene driven by the CaMV35S promoter and terminated by the NOS.

# APPENDIX G

# AGROBACTERIUM TUMEFACIENS MEDIATED TRANSIENT EXPRESSION OF APTAMERS IN BEAN, SWEET ORANGE AND TOMATO LEAVES

1. Insert Preparation:

*E. coli* pGZ7.5/DH5α (YPASYMQ in pBI221 vector) and pGZ7.6/DH5α (HPYTFLN in pBI221 vector) were streaked out on LB agar plates with ampicillin at a final concentration of 100 μg/ml. Single colonies were inoculated into 2 ml of LB with ampicillin at a final concentration of 100 μg/ml and the culture was incubated at 37°C in a rotary shaker at a speed of 220 rpm overnight. Plasmid DNA was prepared and dissolved in 30 μl of TE (pH 8.0). The DNA quality was tested by 0.7% agarose gel electrophoresis. The estimated DNA concentration was 1 μg/μl.

The plasmid DNAs were digested by *Eco*RI as follows: 20 μl of plasmid DNA, 4 μl of 10X REact Buffer 3, 1 μl of *Eco*RI, 5 μl of RNase (200 μg/ml) and 10 μl of $dH_2O$. The digestion mixture was incubated at 37°C for 1 hr. 500 μl of TE (pH 8.0) was added after reaction. Phenol/Chloroform treatment was performed to remove protein. The DNA was precipitated by adding 1 ml of 95% ethanol and 50 μl of 3M sodium acetate (pH 5.3) at -20°C overnight. The precipitate mixture was centrifuged at 10,000 rpm for

15 min at 4°C. The pellet was washed by 70% ethanol, briefly air-dried and re-dissolved in 20 µl of TE (pH 8.0). The DNA concentration was measured by 0.7% agarose gel electrophoresis. The concentration of both inserts was adjusted to 1 µg/µl.

2. Vector Preparation:

*E. coli* pYD40.1/DH5α (*pthA* full-length gene in pGZ6.4 binary vector) and pYD40.2/DH5α (pGZ6.4 binary vector only) were grown, DNA extracted, and digested with *Eco*RI as described in Appendix G1. The concentration of both pYD40.1/*Eco*RI and pYD40.2/*Eco*RI was adjusted to 1 µg/µl.

pYD40.1/*Eco*RI and pYD40.2/*Eco*RI fragments were treated with Shrimp Alkaline Phosphatase (SAP) to eliminate self-ligation. The reaction was set up as follows: 20 µl of DNA, 5 µl of 10X buffer, 1 µl of Shrimp Alkaline Phosphatase (1:1 fresh diluted with 1X Dilution Buffer) and 24 µl of distilled water. The mixture was incubated at 37°C for 1hr, then heated at 65°C for 20 min to inactivate Shrimp Alkaline Phosphatase.

The ligation reactions were set up as follows:

| | |
|---|---|
| Insert (pGZ7.5/*Eco*RI or pGZ7.6/*Eco*RI) | 4 µl |
| Vector (pYD40.1/*Eco*RI or pYD40.2/*Eco*RI) | 2µl |
| 5X ligation buffer | 2 µl |
| T4 DNA ligase | 1 µl |
| $dH_2O$ | 1 µl |

The mixture was incubated at 16°C overnight. There were four sets of ligation: pGZ7.5/*Eco*RI + pYD40.1/*Eco*RI, pGZ7.5/*Eco*RI + pYD40.2/*Eco*RI, pGZ7.6/*Eco*RI + pYD40.1/*Eco*RI and pGZ7.6/*Eco*RI + pYD40.2/*Eco*RI.

3. Transformation to *E. coli* DH5α:

50 µl of thawed *E. coli* DH5α competent cells were transferred to a 1.5 ml microcentrifuge tube, mixed with 5 µl of overnight ligation product and incubated on ice for 10 min (no shaking). The mixture was heat shocked for 1 min at 42°C and put back on ice for 5 min. One ml of SOBG medium was added and the mixture was incubated at 37°C in a rotary shaker in a speed of 220 rpm for 45 min. 150 µl of transformed culture was spread on an LB agar plate with carbenicillin at a final concentration of 50 µg/ml for pBI221 and kanamycin at a final concentration of 50 µg/ml for pGZ6.4. The plate was inverted and incubated at 37°C overnight.

4. Identification of Clones:

Single colonies were inoculated into 2 ml of LB medium with appropriate antibiotics, and the cultures were incubated at 37°C in a rotary shaker at a speed of 220 rpm overnight. Plasmid DNA was prepared and digested by *Eco*RI as follows: 2 µl of plasmid DNA, 2 µl of 10X REact Buffer 3, 1 µl of *Eco*RI, 1 µl of RNase (200 µg/ml) and 14 µl of $dH_2O$. The digestion mixture was incubated at 37°C for 1 hr. The digestion was evaluated by 0.7% agarose gel electrophoresis. Interesting clones with insertions were further tested to check the orientation of the inserts by *Sst*I digestion. There were four possible combinations: pYD40.1 + pGZ7.5 (YPASYMQ aptamer sequence with *pthA*

gene in Ti binary vector), pYD40.2 + pGZ7.5 (YPASYMQ aptamer sequence only in Ti binary vector), pYD40.1 + pGZ7.6 (HPYTFLN aptamer sequence with *pthA* gene in Ti binary vector), and pYD40.2 + pGZ7.6 (HPYTFLN aptamer sequence only in Ti binary vector). Each of the combinations was found during screening and they were named as pGZ8.1, pGZ8.2, pGZ8.3 and pGZ8.4, respectively.

5. Plasmid Mating into *Agrobacterium tumefaciens* GV2260:

Donor strains: *E. coli* DH5α with pGZ8.1, pGZ8.2, pGZ8.3, pGZ8.4 ($Cb^r$ 50 ,$Km^r$ 50)

Recipient strain: *Agrobacterium tumefaciens* GV2260 ($Rif^r$ 75)

Helper strain: *E. coli* HB101/pRK 2073 ($Sp^r$ 50)

Control strains: *E. coli* DH5α with pYD40.1, pYD40.2 ($Km^r$ 50)

*E. coli* strains were inoculated into 2 ml of LB broth with appropriate antibiotics and incubated at 37°C in a rotary shaker in a speed of 220 rpm overnight. *Agrobacterium tumefaciens* GV2260 was inoculated in LB broth with rifomycin at a final concentration of 75 μg/ml and incubated at 28°C in a rotary shaker in a speed of 60 rpm overnight. The overnight cultures were transferred to a 1.5ml microcentrifuge tube and centrifuged at 5,000 rpm at 4°C to collect bacterial cells. The pellet was re-suspended in 100 μl of fresh LB medium. Two-day-old LB agar plates were preferred for the mating process, because they were not too wet or too dry. 100 μl of the recipient strain suspension was dropped onto a marked spot on the LB agar plate and air dried in a

lumina flow hood, then, 100 µl of donor strains suspension was dropped on the same spot and air dried, finally, 100 µl of Helper strain suspension was dropped on the top of same spot and air dried. The plate was incubated at 28°C for 6 hours. The mating mixtures that included pGZ8.1, pGZ8.2, pGZ8.3 and pGZ8.4. Those strains were separately streaked on LB plates with carbenicillin at a final concentration of 50 µg/ml, kanamycin at a final concentration of 50 µg/ml and rifamycin at a final concentration of 75 µg/ml. The mating mixtures that included pYD40.1 and pYD40.2 were separately streak out on LB plates with kanamycin at a final concentration of 50 µg/ml and rifamycin at a final concentration of 75 µg/ml. Plates were inverted and incubated at 28°C overnight.

6. *Agrobacterium tumefaciens* Infiltration into Bean Leaves:

*Agrobacterium tumefaciens* GV2260 strains with pGZ8.1, pGZ8.2, pGZ8.3 or pGZ8.4 were inoculated in 20 ml of YEB medium with carbenicillin at a final concentration of 50 µg/ml, kanamycin at a final concentration of 50 µg/ml and rifamycin at a final concentration of 75 µg/ml. As the positive and negative control, *Agrobacterium tumefaciens* GV2260 strains with pYD40.1 and pYD40.2, respectively, were inoculated in 20 ml of YEB medium with kanamycin at a final concentration of 50 µg/ml and rifamycin at a final concentration of 75 µg/ml. The inoculated culture was incubated at 28°C in a rotary shaker at a speed of 60 rpm overnight. The incubation was stopped when the $OD_{600}$ reached 0.8. The bacterial culture was centrifuged at 5,000 rpm at 4°C and the bacterial cells were re-suspended in an appropriate volume of MMA medium to reach $OD_{600}$=0.5 (for bean). The *Agrobacterium* MMA medium suspension was kept at

room temperature for 1 hr before infiltration. Young California Light Red Kidney bean leaves (about 10 days old) were detached at the same stem and decontaminated with 1% Sodium hypochlorite for 5 min, then washed twice by distilled water for 10 min. The leaves were dried briefly by gently pressed between two layers of 3MM filter paper and transferred to a clean 100 mm petri dish. 10 ml of *Agrobacterium* MMA medium suspension was added. The petri dish was transferred to a vacuum container and the leaves were infiltrated with the *Agrobacterium* suspension under a vacuum of 0.5 mbar for 20 min. The vacuum valve was slowly released to avoid trapping air-bubbles during the *Agrobacterium* infiltration into the leaves. The infiltrated leaves turned a very dark green color. The infiltrated leaves were incubated in a petri-dish with three-layers of 3MM filter paper soaked by distilled water. The petri dish was sealed by parafilm to avoid contamination and kept at 22°C under fluorescent light for 36 hr.

7. *Agrobacterium tumefaciens* Inoculation into Sweet Orange Leaves:

*Agrobacterium tumefaciens* GV2260 constructs were pelleted and re-suspended in MMA medium to reach a final $OD_{600}$ equal to 0.3 (for citrus). Tuberculin (1cc) clinical syringes without needles were used to inject suspensions of *Agrobacterium tumefaciens* GV2260 constructs into the abaxial surface of very young, tender sweet orange leaves. The diameter of the injection spot was around 10 mm. The injection was very gentle to avoid damage to the leaf tissue. The inoculated plants were transferred to a green house kept at 28°C in a high humidity for at least four weeks. Observations were recorded after two weeks.

8. *Agrobacterium tumefaciens* Inoculation into Young Tomato Leaves:

*Agrobacterium tumefaciens* GV2260 constructs were pelleted and re-suspended in MMA medium to reach a final $OD_{600}$=0.3 (for tomato). Inoculations were as for sweet orange. The inoculated tomato plants were transferred to a growth chamber under low light conditions and incubated at 28°C for 24 hr. The symptoms were recorded after 18 hours.

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# BIOGRAPHICAL SKETCH

Mr. Ge Zhao was born on September 25, 1967 in Beijing, China, to Yufeng Zhao (father) and Xingcai Gao (mother). He is the only child in the family. He attended college in Department of Animal Physiology and Biochemistry of China Agricultural University, Beijing, China, from September 1986 to July 1990 and earned a Bachelor of Science degree. After that, he pursued graduate study in Department of Plant Physiology and Biochemistry of China Agricultural University, from September 1990 to July 1993 and earned a Master of Science degree in Biochemistry. Then, he worked as an Assistant Research Fellow in China Agricultural University from September 1993 to February 1994, and then moved to Center of Biotechnology of Institute of Microbiology in Chinese Academy of Sciences as a Research Assistant Fellow till he became a Florida Gator.

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