Development of Polyclonal and Monoclonal Antibody Assays for Detection of Nep1-Like Proteins and Neutralization of Associated Necroses in Plant Tissues

By

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A Thesis
Presented to
The University of Guelph

In partial fulfilment of the requirements for the degree of Doctor of Philosophy in Environmental Biology

Guelph, Ontario, Canada
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ABSTRACT

DEVELOPMENT OF POLYCLONAL AND MONOCLONAL ANTIBODY ASSAYS FOR DETECTION OF NEP1-LIKE PROTEINS AND NEUTRALIZATION OF ASSOCIATED NECROSES IN PLANT TISSUES

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University of Guelph, 2014

*Pythium aphanidermatum* is a detrimental plant pathogen capable of infecting a broad range of hosts, particularly in hydroponic greenhouse systems. Its key factor in pathogenesis is the secreted protein toxin NLP<sub>Pya</sub> belonging to the NLP (Nep1-like protein) family of virulence factors. NLPs are characterized by their ability to trigger necrosis in dicotyledonous plants and their NPP1 domain containing paired cysteines and a central conserved heptapeptide motif ‘GHRHDWE’. In this thesis, anti-NLP antibodies were examined for their ability to detect and neutralize NLPs. Polyclonal anti-NLP<sub>Pya</sub> antiserum was investigated for its ability to bind other NLPs through their conserved regions; the serum bound all NLPs tested with nM affinity. Monoclonal anti-NLP antibodies were raised by immunizing mice with the GHRHDWE heptapeptide. The anti-NLP mAb was tested for binding to NLPs in several different antibody formats (IgM, F(ab’)µ, scFv and IgG); the scFv and IgG bound NLPs with µM to nM affinities. Finally, the abilities of the polyclonal anti-NLP<sub>Pya</sub> serum and monoclonal anti-NLP IgG to neutralize NLP-induced necrosis in plants were evaluated through spot infiltrations in plant leaf tissue. The polyclonal serum was capable of neutralizing necrosis induced by some NLPs, while the anti-NLP IgG was capable of neutralizing necrosis induced by all NLPs tested.
To the countless people whose lives are overshadowed by depression. Keep fighting, no matter what.
ACKNOWLEDGEMENTS

I have had the incredible fortune of being supported and guided by many amazing people throughout my PhD. My graduate school experience presented me with many challenges; it is not a stretch to say that it was the most difficult time in my life. The struggle was made bearable by having wonderful people on whom I could depend.

Chris, thank you for being my advisor. You have always understood that my graduate student experience was as much about personal growth as it was about growth as a scientist and an academic professional. You gave me the space I needed to mature as an independent person, and the motivation to persevere through a difficult journey. Thank you for your patience and wisdom.

Thank you to my JCH labmates, for sharing with me your collectively immense knowledge and diverse perspectives. Rashidah, your optimism and determination continue to inspire me. Haifeng, your expertise and generosity were greatly encouraging. Mike, your discerning eye strengthened my thesis as a work of science and me as a scientist. Sara, Erin and John, thank you for the much-needed laughs!

My friends have been an endless source of joy and revitalization. Karley, Dave, Waseem, Aneta, and Filomena: you taught me that family and friends can be one and the same. Scout and Marley, thank you for your unconditional love and 5am wakeup calls. Omar, thank you for rekindling hope and fun.

Mom and Carlos, you have been my twin pillars of support throughout the entirety of this thesis. Thank you for inspiring me, believing in me, loving me, and always helping me to do my best. Thank you for being my parents.
# Development of Polyclonal and Monoclonal Antibody Assays for Detection of Nep1-Like Proteins and Neutralization of Associated Necroses in Plant Tissues

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<tr>
<td>2x YT</td>
<td>Yeast tryptone medium pH 7.2 containing 16 g/L tryptone, 10 g/L yeast extract and 5 g/L NaCl</td>
</tr>
<tr>
<td>4-HBA</td>
<td>4-hydroxybenzoic acid</td>
</tr>
<tr>
<td>5-FAM</td>
<td>5-carboxy fluorescein</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction coefficient, measured in M-1 cm-1</td>
</tr>
<tr>
<td>A&lt;sub&gt;280&lt;/sub&gt;</td>
<td>Absorbance read at 280 nm</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABC</td>
<td>Arabidopsis basic chitinase signal sequence</td>
</tr>
<tr>
<td>AFP</td>
<td>Anti-fungal peptides</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetracetic acid</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BGG</td>
<td>Bovine gamma globulin</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>bp</td>
<td>DNA base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>C&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Constant domain of antibody heavy chain</td>
</tr>
<tr>
<td>CI-ELISA</td>
<td>Competitive inhibition ELISA</td>
</tr>
<tr>
<td>CI-FPIA</td>
<td>Competitive inhibition FPIA</td>
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<tr>
<td>C&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Constant domain of antibody light chain</td>
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<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Delbucco’s modified eagle medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<tr>
<td>dsFv</td>
<td>Double stranded variable fragment</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetracetic acid</td>
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<td>Abbreviation</td>
<td>Unabbreviated Term</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment; antigen binding region</td>
</tr>
<tr>
<td>Fab(\mu)</td>
<td>Fragment; antigen binding region; (\mu)-chain specific</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment; crystallisable region</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
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<tr>
<td>FPIA</td>
<td>Fluorescence polarization immunoassay</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>G+C content</td>
<td>Guanidine plus cytosine content</td>
</tr>
<tr>
<td>(G4S)(3)</td>
<td>(Glycine (x4), Serine) (x3) linker sequence</td>
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<tr>
<td>GHRH(D)H(W)E</td>
<td>Glycine-histidine-arginine-histidine-aspartate-tryptophan-glutamate</td>
</tr>
<tr>
<td>GRAVY</td>
<td>Grand average of hydropathy</td>
</tr>
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<td>HAMA</td>
<td>Human anti-mouse antibodies</td>
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<td>HBS-EP(^+)</td>
<td>10mM HEPES buffer pH 7.4 containing 150mM NaCl, 3mM EDTA and 0.05% P20</td>
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<td>HC</td>
<td>Antibody heavy chain</td>
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<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
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<tr>
<td>HIV-1</td>
<td>Human type 1 immunodeficiency virus</td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitive response</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>HT</td>
<td>Human transferrin</td>
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<td>HT supplement</td>
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<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
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<td>Immunoglobulin</td>
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<td>II</td>
<td>Instability index</td>
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<tr>
<td>IMAC</td>
<td>Immobilized metal ion affinity chromatography</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-(\beta)-D-1 thiogalactopyranoside</td>
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<td>Intravenous</td>
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<td>kDa</td>
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<td>Abbreviation</td>
<td>Unabbreviated Term</td>
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</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
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<td>LB</td>
<td>Lysogeny broth pH 7.5 containing 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl</td>
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<td>LB-carb</td>
<td>Lysogeny broth plus carbenicillin</td>
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<td>LC</td>
<td>Antibody light chain</td>
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<td>Limit of blank</td>
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<td>LoD</td>
<td>Limit of detection</td>
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<tr>
<td>M</td>
<td>Molar; moles per litre</td>
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<td>M13</td>
<td>Strain of filamentous phage</td>
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<td>MA</td>
<td>Matrix-adjusted</td>
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<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MAP kinase</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>mAU</td>
<td>Milliabsorbance units</td>
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<tr>
<td>MES</td>
<td>1-(N-morpholino)ethanesulfonic acid</td>
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<td>mIgM</td>
<td>Membrane-bound immunoglobulin M</td>
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<tr>
<td>mP</td>
<td>Millipolarization units</td>
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<td>MS∅</td>
<td>Murashige Skoog Basal Medium</td>
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<tr>
<td>MS-NLP_Pya</td>
<td>MS∅ amended with NLP_Pya</td>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
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<td>MWCO</td>
<td>Molecular weight cut-off</td>
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<tr>
<td>N nucleotides</td>
<td>Non-coded nucleotides</td>
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<td>nAb</td>
<td>Neutralizing antibody</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<td>NBT-BCIP</td>
<td>Nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>Nep1</td>
<td>Necrosis- and ethylene-inducing protein 1</td>
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<td>N-glycans</td>
<td>N-linked glycans on the endoplasmic reticulum</td>
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<td>Ni-NTA</td>
<td>Nickel nitrilotriacetic acid</td>
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<td>NLP</td>
<td>Nep1-like protein</td>
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<td>NLP_Bc1</td>
<td>NLP1 from <em>Botrytis cinerea</em></td>
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<td>NLP2 from <em>Botrytis cinerea</em></td>
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<td>NLP from <em>Fusarium oxysporum</em> f. sp. <em>erythroxyl</em></td>
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<td>NLP from <em>Pectinobacterium carotovora</em> subsp. <em>carotovora</em></td>
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<td>NLP_Pya</td>
<td>NLP from <em>Pythium aphanidermatum</em></td>
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<td>NLP_Sscl</td>
<td>NLP from <em>Sclerotinia sclerotiorum</em></td>
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<tr>
<td><strong>ABBREVIATION</strong></td>
<td><strong>UNABBREVIATED TERM</strong></td>
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<td>NLP&lt;sub&gt;Ssc1&lt;/sub&gt;</td>
<td>NLP1 from <em>Sclerotinia sclerotiorum</em></td>
</tr>
<tr>
<td>NLP&lt;sub&gt;Ssc2&lt;/sub&gt;</td>
<td>NLP2 from <em>Sclerotinia sclerotiorum</em></td>
</tr>
<tr>
<td>NLP- heptapeptide</td>
<td>Central conserved seven amino acid motif from NLPs comprising</td>
</tr>
<tr>
<td></td>
<td>GHRHDWE</td>
</tr>
<tr>
<td>NPP1 domain</td>
<td>Necrosis-inducing <em>Phytophthora</em> protein domain consisting of NLP-heptapeptide and one or two cysteine pairs</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand white rabbit</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>P nucleotides</td>
<td>Palindromic nucleotide sequences</td>
</tr>
<tr>
<td>P19</td>
<td>Suppressor of gene silencing protein in plants</td>
</tr>
<tr>
<td>P20</td>
<td>Polysorbate 20 detergent</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PAL</td>
<td>Phenylalanine ammonium-lyase</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PaNie</td>
<td><em>Pythium aphanidermatum</em> necrosis-inducing elicitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline pH 7.4 containing 137mM NaCl, 2.7mM</td>
</tr>
<tr>
<td></td>
<td>KCl, 10mM Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt; and 1.8mM KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>PBS+P&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Phosphate buffered saline plus 0.05% P20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>Pfū</td>
<td>DNA polymerase engineered from <em>Pyrococcus furiosus</em></td>
</tr>
<tr>
<td>pH</td>
<td>Power of hydrogen</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PO</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PPO</td>
<td>Polyphenol oxidase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>PVX</td>
<td>Potato virus X</td>
</tr>
<tr>
<td>R&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum analyte binding capacity</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RU</td>
<td>Response units</td>
</tr>
<tr>
<td>scFv</td>
<td>Single chain variable fragment</td>
</tr>
<tr>
<td>sdAb</td>
<td>Single-domain antibodies</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Unabbreviated Term</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate poly-acrylamide electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sIgM</td>
<td>Secretory immunoglobulin M</td>
</tr>
<tr>
<td>SLC</td>
<td>Surrogate light chain</td>
</tr>
<tr>
<td>SOC</td>
<td>Super optimal catabolite repression medium pH 7.0 containing 20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO₄, 10 mM MgCl₂ and 20 mM glucose</td>
</tr>
<tr>
<td>Taq</td>
<td>DNA polymerase engineered from <em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TC sup</td>
<td>Hybridoma tissue culture supernatant</td>
</tr>
<tr>
<td>T-DNA</td>
<td><em>Agrobacterium</em>-transferred DNA</td>
</tr>
<tr>
<td>Ti plasmid</td>
<td>Tumour-inducing plasmid</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TMV</td>
<td>Tobacco mosaic virus</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethyl-propane-1,3-diol</td>
</tr>
<tr>
<td>TSP</td>
<td>Total soluble protein</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyoxyethylene (20) sorbitan monolaurate</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V₉H</td>
<td>Variable domain of antibody heavy chain</td>
</tr>
<tr>
<td>V₉H₉H</td>
<td>Camelid variable chain heavy fragment</td>
</tr>
<tr>
<td>vir</td>
<td>Virulence</td>
</tr>
<tr>
<td>V₉L</td>
<td>Variable domain of antibody light chain</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
</table>
1. **General Introduction and Research Objectives**

*Pythium aphanidermatum* is a detrimental plant pathogen capable of infecting a broad range of hosts around the world (van der Plaats-Niterink, 1981). *Pythium* infections are especially prevalent in hydroponic systems, causing 80% of greenhouse epidemics with the genera *Phytophthora*, and *Fusarium* (Schuerger, 1998). *P. aphanidermatum* is estimated to be the most common *Pythium* species found in Ontario (Zheng et al., 2000). One of the key factors of *P. aphanidermatum* pathogenesis is the production of a proteinaceous toxin known as NLP<sub>Pya</sub>. Since NLP<sub>Pya</sub> is the only known toxin produced by *P. aphanidermatum*, this protein makes an attractive target for neutralization as a means to attenuate *P. aphanidermatum*-induced disease.

NLP<sub>Pya</sub> is part of the growing NLP (Nep1-like protein) family of proteinaceous virulence factors (Ottmann et al., 2009). NLPs are 25-27 kDa proteins characterized by their ability to trigger necrosis in dicotyledonous plants. Their conserved NPP1 domain consists of two or four cysteine residues and a central conserved heptapeptide motif ‘GHRHDWE’ (Gijzen and Nürnberger, 2006). NLPs have been identified in a broad range of phytopathogenic hosts including bacteria, oomycetes and fungi. To date, research on NLPs has focused on elucidation of their molecular and physiological mechanisms and isolation or identification of new NLP species from their originating organisms or via their genetic coding sequences (Ottmann et al., 2009; Küfner et al., 2009; Dong et al., 2012). To the knowledge of this author, neutralization of the necrotic activity of NLPs has not been investigated.

In the immune system, antibodies recognize and selectively bind specific molecular targets to invoke an immune response against them (Goldsby et al., 2003). In a
laboratory setting, antibodies can be raised against specific antigens and utilized for a myriad of applications (Yau et al., 2003). A typical antibody application is the detection and quantification of chemicals, such as environmental contaminants (Rubio et al., 2003). Antibodies also have numerous medical applications, such as the treatment of human afflictions like toxic snakebites or infectious viruses like Hepatitis C or HIV-1 with neutralizing antibodies (Bortnik and Rozanov, 1979; Di Lorenzo et al., 2011; Kwong et al., 2011). Neutralizing antibodies could presumably be utilized to combat diseases in plants, although this concept remains largely unexplored.

Previously, in the Hall laboratory, NLP$_{Py}$ was cloned from *Pythium aphanidermatum* genomic DNA for expression in *E. coli*; rabbits were immunized with recombinant NLP$_{Py}$ and the resulting serum was shown to have a high anti-NLP$_{Py}$ titre (Fjällman, 2008). The main objectives of this thesis were to raise NLP-specific monoclonal antibodies and to investigate the detection and quantification of NLPs and the neutralization of NLP-induced necrosis. Therefore, the following hypotheses and research objectives were proposed.

**Hypothesis:** Based on their conserved structures, antiserum raised against one NLP, NLP$_{Py}$, will have the ability to bind other NLPs

This hypothesis was investigated in Chapter 3 by the following means:

- Three additional NLPs were cloned for expression and study, i.e., the archetype NLP$_{Fo}$ from *Fusarium oxysporum* f. sp. *erythroxyli*, the unstudied NLP$_{Bha}$ from *Bacillus halodurans* and the hypothetical NLP$_{Ssc}$ from *Sclerotinia sclerotiorum*
• Rabbit anti-NLP\textsubscript{pya} serum was investigated in immunoblot and ELISA formats to determine if it would bind the three other NLPs

• Binding to all four NLPs was quantified using a CI-ELISA format

**Objective:** To develop a polyclonal anti-NLP assay capitalizing on this putative cross-reactivity to detect multiple NLPs

**Hypothesis:** a cross-reactive monoclonal anti-NLP antibody could be raised by immunization with a conserved NLP structure, i.e., the NLP-heptapeptide ‘GHRHDWE’

This hypothesis was investigated in Chapter 4 by the following means:

• Mice were immunized with the NLP-heptapeptide for the purpose of raising a monoclonal antibody capable of binding several NLPs

• Several anti-NLP mAbs were isolated and the best clone was evaluated in several antibody formats (IgM, F(ab’\textsubscript{2}), scFv, IgG) to explore the advantages and disadvantages of these various formats

• Binding to all four NLPs was quantified using CI-ELISA format

**Objective:** To develop a monoclonal anti-NLP assay with similar affinities for all NLPs

**Hypothesis:** Due to their cross-reactivity, the polyclonal anti-NLP\textsubscript{pya} rabbit serum and the monoclonal anti-NLP IgG will neutralize NLP activity in plants

This hypothesis was investigated in Chapter 5 by the following means:

• The necrotic effect of NLPs on plant tissue was demonstrated via spot infiltrations with the purified proteins
Neutralizing capabilities of the polyclonal anti-NLP$_{pya}$ serum were evaluated via spot co-infiltrations with purified NLPs and the serum.

Neutralizing capabilities of the anti-NLP IgG were evaluated via spot co-infiltrations with purified NLPs and the purified mAb.

**Objective:** To evaluate the abilities of polyclonal and monoclonal anti-NLP antibodies to neutralize NLP-induced necroses.

**Hypothesis:** Due to its high affinity for NLP$_{pya}$, the polyclonal anti-NLP$_{pya}$ rabbit serum can be used to detect and quantify NLP$_{pya}$ in the nutrient solutions of *P. aphanidermatum*-infected hydroponic plants.

This hypothesis was investigated in Chapter 5 by the following means:

- Hydroponic plant growth system was inoculated with *P. aphanidermatum* and the infected growth media was assayed for NLP$_{pya}$.
- NLP$_{pya}$ in the nutrient solutions of *P. aphanidermatum*-infected hydroponic plants was quantified using polyclonal anti-NLP$_{pya}$ serum in a CI-ELISA format.

**Objective:** To evaluate the ability of the polyclonal assay developed in Chapter 3 to detect and quantify NLP$_{pya}$ in the hydroponic solution following a *P. aphanidermatum* infection.
2. **Literature Review**

2.1. **Antibodies**

2.1.1. **Conventional antibodies**

Antibodies or immunoglobulins are proteins capable of selectively binding to specific molecular targets, a.k.a. antigens. They are produced by the B lymphocytes of mammalian immune systems as part of the humoral immune response. In the immune system, antibodies recognize and bind foreign or ‘non-self’ antigens to invoke an immune response against them (Goldsby et al., 2003). In a laboratory setting, antibodies can be raised against specific antigens and utilized in experimental techniques such as enzyme-linked immunosorbent assays (ELISAs) or immunoblotting (Yau et al., 2003).

There are five classes of immunoglobulins: IgG, IgA, IgM, IgD and IgE. IgG antibodies are the most abundant class in serum and are commonly used in diagnostic procedures. IgG have a common homodimeric structure which consists of two identical light (L) polypeptide chains and two identical heavy (H) polypeptide chains with molecular weights of about 25 000 and 50 000 Da, respectively. Each light chain is bound to the heavy chain covalently though a disulfide bond and noncovalently through ionic, hydrogen and hydrophobic bonds to form a H-L heterodimer. The two H-L heterodimers are bound together at their heavy chains in a similar fashion. Secreted IgM usually exists as a pentamer and IgA has been known to exist as a dimer or trimer (see Figure 2.1) (Maynard and Georgiou, 2000).
Figure 2.1. General structures of the five major classes of secreted antibody.

Light chains are shown in green; heavy chains are shown in blue; carbohydrates are shown in yellow; J chains and disulfide bonds are indicated with black lines. Diagram modified from Actor, 2006.

Antigen binding is mediated by the variable regions of the heavy and light chains, $V_H$ and $V_L$, which can be further divided into different complementarity-determining regions (CDRs). The antigen binding region, known as the paratope, interacts with a specific epitope on an antigen. Specificity of an antibody is determined by the sequences of amino acids present in the CDRs of the paratope (Goldsby et al., 2003).
2.1.1.1. Immunoglobulin M (IgM)

Immunoglobulin M, or IgM, is the first antibody produced in the primary humoral immune response and accounts for approximately 5 to 10% of total serum immunoglobulin (Goldsby et al., 2003). The secretory form of IgM is a 900kDa pentamer consisting of five monomers attached by their Fc regions plus an additional Fc-linked polypeptide known as the joining chain or J chain (see Figure 2.2). IgM only exists as a monomer in its membrane-bound form on the B cell surface, forming the B cell receptor (BCR). Each 180kDa IgM monomer consists of a \( \mu \) heavy chain with four \( C_\mu \) regions and a \( V_\mu \) region, and \( \kappa \) or \( \lambda \) light chain with one \( C_\kappa \) and one \( V_\kappa \) region (Anelli and van Anken, 2013).

IgM variable regions are synthesized during B lymphocyte development through the rearrangement of germ-line multigene families. The \( V_\mu \) is formed through rearrangement of \( V_\mu, D_\mu \) and \( J_\mu \) genes which then join with a \( C_\mu \) gene to form the HC; likewise, the \( V_\kappa \) is formed by \( V_\kappa \) and \( J_\kappa \) gene rearrangement which then join with a \( C_\kappa \) or \( C_\lambda \) gene to form the LC. The numbers of gene segments in these multigene families vary between species (see Table 2.1). The combinatorial joining of these multiple germ-line gene segments generates antibody diversity, as do the junctional flexibility and addition of P- and N-nucleotides inherent to this process. Based on the number of genes in these multigene families, the number of potential antibody gene segments produced in humans is \( 2.64 \times 10^6 \) and in mice is \( 2.41 \times 10^6 \). Mature B cells undergo the \textit{in vivo} affinity maturation process of hypersomatic mutation, then class-switching from \( \mu \) to \( \gamma \), \( \alpha \), \( \delta \) and \( \varepsilon \) isotypes (Goldsby et al., 2003)
Figure 2.2. Schematic representation of membrane bound IgM and secretory IgM. Lymphocytes display membrane bound IgM (mIgM), shown on the left, on their surface as the core part of the BCR. Once committed to the plasma cell stage they produce secretory IgM (sIgM) in either “pentameric” form, shown on the right, or “hexameric” form (not shown). HCs and LCs consist of the various Ig-fold domains $V_H$, $C_{H1-4}$, $V_L$, and $C_L$ that are color-coded as indicated. Glycans and intra- and inter-subunit disulfide bonds between HCs, LCs, and J-chain are depicted. Note that the Ig-$\mu_m$ HC (in mIgM) differs from the Ig-$\mu_s$ HC (in sIgM) in their C-termini, having a transmembrane domain (TMD) or, respectively, a cysteine-containing tail piece (TP) as indicated. Diagram modified from Anelli and van Anken, 2013.
IgM antibodies are produced before B cells undergo affinity maturation and as such, have relatively low affinities compared to their class-switched successors. The pentameric structure of secreted IgM allows avidity to compensate for this lack of affinity (Boes, 2000). Due to its high valency, the IgM pentamer is very efficient at agglutinating small particles with repeating epitopes such as viruses. This makes it an effective reagent for the neutralization of viral particles such as the influenza virus and for detection of viruses like Epstein-Barr and strains of Dengue (Jayasekera et al., 2007; Schaade et al., 2001; Jahanshahi et al., 2014). IgM is also quite efficient at complement activation, as this function requires two Fc regions in close proximity and the IgM pentamer has five. Its high molecular weight decreases its ability to diffuse into tissues; this is mediated by the J chain’s ability to bind transport receptors, enabling the IgM molecule to traverse epithelial cell linings (Johansen et al., 2000).

<table>
<thead>
<tr>
<th>Gene Locus</th>
<th>Human</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH Genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>51</td>
<td>134</td>
</tr>
<tr>
<td>D</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>J</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>VL λ Genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>31 (Ψ)</td>
<td>3</td>
</tr>
<tr>
<td>J</td>
<td>4 (Ψ)</td>
<td>4 (1Ψ)</td>
</tr>
<tr>
<td>C</td>
<td>7 (Ψ)</td>
<td>4</td>
</tr>
<tr>
<td>VL κ Genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>40</td>
<td>85</td>
</tr>
<tr>
<td>J</td>
<td>5</td>
<td>5 (1Ψ)</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Table 2.1. Number of germline gene segments found in each immunoglobulin multigene locus in humans and mice. (Ψ) denotes presence or number of pseudogenes within a locus*
2.1.1.2. Immunoglobulin G (IgG)

Immunoglobulin G, or IgG, is the most abundant antibody class in serum, comprising approximately 80% of total serum immunoglobulin. IgG exists in serum as a 150 kDa monomer with γ heavy chain consisting of three C\textsubscript{H} regions, a V\textsubscript{H} region and a hinge region, and κ or λ light chain with one C\textsubscript{L} and one V\textsubscript{L} region. Humans and mice each possess four IgG subclasses differing from one another by the size of their hinge regions and the number and position of interchain disulfide bonds between the heavy chains; these subclasses are denoted IgG\textsubscript{1}-IgG\textsubscript{4} in humans and IgG\textsubscript{1}, IgG\textsubscript{2a/b} and IgG\textsubscript{3} in mice. In humans, these structural differences afford the IgG classes different abilities such as crossing the placenta (IgG\textsubscript{1}, 3, 4), activating complement (IgG\textsubscript{1}, 2, 3), and mediating opsonization (mainly IgG\textsubscript{1} and 3) (Goldsby et al., 2003).

IgG antibodies are produced by differentiated B lymphocytes, meaning they have undergone somatic hypermutation and are affinity matured. As such, IgG have more diverse sequences and much higher affinities than their IgM precursors. As discussed in 2.1.1.1, the combinatorial joining of the human V-D-J-C and V-J-C multigene families can potentially produce $2.64 \times 10^6$ antibody gene segments (Goldsby et al., 2003). Somatic hypermutation occurs at a rate of $10^{-3}$/basepair/generation, or approximately $10^6$-fold greater than the normal rate of mutation, which increases this potential to $10^{10}$ antibody gene segments (Teng and Papavasiliou, 2007). The maximum estimated affinity (affinity ceiling) of an IgG produced under physiological conditions is $10^{10}$ M\textsuperscript{-1}, while IgM antibodies exhibit an affinity ceiling of approximately $10^7$ M\textsuperscript{-1} (Foote and Eisen, 1995; Fan and Karush, 1984).
Due to their high affinities and many varied biological functions, IgG are the antibodies of choice for most applications. For example, 28 of the 30 monoclonal therapeutic antibodies currently approved for clinical use by the United States Food and Drug Administration (FDA) and/or the European Medicines Agency are IgG isotype (Reichert, 2012; U.S. Food and Drug Administration, 2013b).

2.1.2. Recombinant antibodies

Antibodies can be raised to exhibit specific binding to a desired target molecule. Using recombinant DNA technology, the genes for antibodies can be manipulated to create novel antibody fragments such as single chain variable fragments (scFv), double stranded variable fragments (dsFv), camelid heavy chain variable fragments (V_{H}H), or even whole chimeric antibodies (see Figure 2.3) (Maynard and Georgiou, 2000).

2.1.2.1. Chimeric and humanized IgG antibodies

The majority of therapeutic IgG are murine in nature, meaning their efficacy is limited by their ability to elicit an immune response in the form of human anti-mouse antibodies (HAMA). One way to circumvent this challenge is to use recombinant DNA technology to eliminate the most immunogenic portions of the antibody (Maynard and Georgiou, 2000). To reduce the immune response generated by a non-human Fc region, murine variable heavy and light fragments can be cloned with human constant regions to create a chimeric IgG. Using this method, an antibody’s immunogenicity can be reduced by an estimated 90% (Adair, 1992). Further reduction of immunogenicity can be achieved by grafting only the murine CDRs on to a human framework scaffold, creating a fully humanized IgG. This reduces the amount of murine material from 30% to 3% of the
antibody’s structure, but does not fully eliminate the risk of HAMA (Jones et al., 1986). Humanization can also result in a drastic reduction in IgG affinity, although this reduction may be reversed through targeted mutagenesis of select framework region amino acids (Foote and Winter, 1992).

Figure 2.3. Schematic showing various antibody fragments of biotechnological and clinical interest. Each block represents one antibody domain with a characteristic immunoglobulin fold. Black bars are interchain disulfide bonds (horizontal) or intradomain linkages (vertical); curved lines are genetically engineered peptide linkers. Diagram modified from Maynard and Georgiou, 2000.
2.1.2.2. Single chain variable fragment (scFv)

Using recombinant DNA techniques, the variable heavy and light fragments of an immunoglobulin can be isolated and cloned without the constant regions to create an Fv antibody (fragment variable region). In this formation the $V_H$ and $V_L$ are held together by noncovalent interactions only, and as such are prone to disassociation and aggregation. Introduction of a short polypeptide linker fuses the variable regions covalently to create an scFv antibody (Maynard and Georgiou, 2000). scFv are relatively small molecules (26-27 kDa) compared to their full-length progenitors, thus affording them superior pharmacokinetic properties without a concomitant loss of affinity (Furrer et al., 2009). Since they lack an Fc region scFv are much less immunogenic than full-length antibodies, making them suitable agents for a variety of therapeutics (Monnier et al., 2013).

The main disadvantage of the scFv antibody format is its instability relative to full-length or Fab antibodies. scFv can be engineered for better stability using a variety of cloning methods, the least invasive of which is to modify the polypeptide linker. This can be accomplished by altering the order in which the variable regions are linked ($V_H$-linker-$V_L$ or $V_L$-linker-$V_H$), the length of the linker, and its composition from the typical $(G_4S)_3$ sequence (Maynard and Georgiou, 2000). scFv instability generally arises from the presence or exposure of ‘problematic residue(s)’ such as hydrophobic amino acids that were previously hidden by the constant regions, hydrophilic amino acids in a framework region $\beta$-sheet or hydrophilic amino acids at the normally hydrophobic interface between the $V_H$ and $V_L$, any of which can lead to protein unfolding or intermolecular aggregation. Problematic residues interfering with protein folding or contributing to aggregation can be substituted through site-directed mutagenesis, although identification of such residues
can be arduous (Chowdhury and Vasmatzis, 2002). Random mutagenesis of framework regions and subsequent screening of resulting clones can improve scFv stability without prior identification of problematic residues. Another more intensive approach is the transplantation of all CDRs to an acceptor framework scaffold possessing suitable biophysical properties. All of these alteration methods are labour-intensive and risk a change in scFv affinity along with stability (Borras et al., 2010).

2.1.3. Antibody expression systems

There are a number of different systems available for antibody expression including bacterial, yeast, plant, insect, mammalian, and in vitro translation systems. There are different problems associated with the expression of each individual antibody or antibody fragment due to their vast sequential and structural differences. Most expression systems must be tailored to the molecule being produced to optimize expression yield and biological activity of the protein product (Verma et al., 1998).

2.1.3.1. Expression in bacteria

Antibody expression is frequently carried out in bacteria, most notably in *Escherichia coli*, because this system is efficient and inexpensive. Transformation of *E. coli* with foreign genes is simple and the transformed cells are able to grow at a very fast rate to produce large quantities of protein in a short period of time. However, there are some drawbacks associated with the *E. coli* system due to the differences between prokaryotic and eukaryotic protein expression. *E. coli* are incapable of glycosylating proteins, meaning whole IgG molecules cannot be expressed in this system since the CH2 domain requires glycosylation (Verma et al., 1998). As the antigen binding parts of an
antibody are typically not glycosylated, *E. coli* is quite effective in expressing antibody fragments such as Fab or scFv (Skerra, 1993). The cytoplasm of bacteria is a reducing environment which can result in the formation of insoluble inclusion bodies consisting of unfolded protein (Verma et al., 1998). Thus, any antibody produced in a bacterial system may need to be refolded after expression or engineered with a leader peptide to target it to the periplasm (Huston et al., 1995; Verma et al., 1998). Once outside the cell, oxidation of the protein’s cysteine thiols into disulfide bonds can occur and the antibody can assume its proper tertiary structure (Skerra and Plückthun, 1988).

The quantity of protein produced using a bacterial system varies depending on the individual antibody being expressed. Expression studies of 206 different Fab clones from the HuCAL library showed an average yield of 10 mg per L culture volume, although some clones yielded up to 30 mg per L culture volume (Knappik and Brundiers, 2009). There are also many factors that must be considered in regards to the growth of the expression host itself, such as where in the cell the protein is found, and optimal growth conditions for the bacterial strain (Verma et al., 1998). Zarschler et al. (2013) investigated the use of a double mutant *E. coli* strain defective in both thioredoxin (*trx*B) and glutathione (*gor*) pathways, enabling it to produce disulfide bonds in the cytoplasm. The strain was expressed in combination with an enzyme-based glucose release system to obtain sdAb yields of up to 200 mg per L culture volume (Zarschler et al., 2013).

2.1.3.2. Expression in plants

A large-scale eukaryotic approach to antibody expression is the production of antibodies in plants (biopharming). A plant expression system is advantageous in that production costs are low and maintenance does not differ from the growth of any other
crop (Fischer et al., 2003). Plants are able to produce full-size antibodies that are properly folded and, for the most part, correctly glycosylated (Cabanes-Macheteau et al. 1999; Chargelegue et al., 2000). Expression of antibodies in transgenic plants with humanized N-glycan profiles has also been successful (Gomord et al., 2010). In addition to full-size antibodies, plants have also been demonstrated to produce antibody fragments such as Fab, scFv and dsFv (Fischer et al., 2003).

Expression of antibodies in plants can be achieved through stable or transient transformation systems. Stable expression typically refers to the production of a homozygous transgenic plant line, a sustainable process that can take months or years to complete. By contrast, transient expression refers to the short-term process by which the leaves of young plants are transformed with the gene(s) of interest and harvested several days later (Garabagi et al., 2012b). In both cases, transformation can be accomplished with Agrobacterium tumefaciens, a bacterial plant pathogen capable of modifying the genome of its hosts through the transfer of DNA (T-DNA) from its own tumour-inducing (Ti) plasmid. For antibody expression, the nonessential oncogene portion of the Ti-plasmid is replaced with the gene(s) of interest while the vir region necessary for DNA transfer is kept intact, creating a disarmed vector. This renders the A. tumefaciens incapable of causing tumours in the host plant and instead causes it to transfer the genes for antibody expression to the plant genome. The Ti-plasmid can be further modified to a helper plasmid by removing the T-DNA, oncogene and opine gene regions and leaving only the vir region. The helper plasmid is transformed in tandem with a binary vector containing T-DNA border repeats and the gene(s) of interest, and the Vir proteins then act on the binary vector to integrate the gene(s) of interest into the host plant genome.
(Meyers et al., 2011). The binary vector system is more streamlined than the use of a disarmed vector, and can yield IgG in the order of 100 to 500 mg per kg plant matter (J.C. Hall, personal communication). Transformation for transient expression can also be accomplished through the use of deconstructed viral vectors such as potato virus X (PVX) or tobacco mosaic virus (TMV) (Qutob et al., 2002; Meyers et al., 2011).

2.1.4. Antibody applications

The specificity and versatility of antibodies has given rise to many diverse applications for these proteins. They are invaluable research and diagnostic tools, providing the means to detect and quantify trace amounts of chemicals and contaminants (Yau et al., 2003). The largest market for antibodies is the pharmaceutical market, where antibodies are being developed to treat a myriad of human diseases and disease symptoms. To date, 28 monoclonal therapeutic antibodies and two monoclonal antibody-drug conjugates are approved for clinical use by the FDA and/or the European Medicines Agency, with hundreds more in development and review (Reichert, 2012; U.S. Food and Drug Administration, 2013a; U.S. Food and Drug Administration, 2013b). The currently approved antibodies treat forms of cardiovascular disease and transplant rejection, autoimmune diseases such as lupus, rheumatoid arthritis and Crohn’s disease, and types of cancer such as breast, colorectal, Non-Hodgkin’s lymphoma and certain leukemias (Reichert, 2012). Antibody treatments for the deteriorative diseases multiple sclerosis and Alzheimer’s disease are currently in development (Sorenson, 2008; Demattos, 2012).

Treatment of plant diseases has also been explored by utilizing antibodies to deliver antimicrobial agents. In 2004, Peschen et al. reported the use of antifungal
peptides (AFP) linked to a scFv specific for cell wall-bound proteins of *Fusarium graminearum*. They found that the fusion proteins tested were able to significantly inhibit mycelial growth of *F. graminearum* and *F. oxysporum* f. sp. *matthiolae* (Peschen et al., 2004). Growth of plant pathogens can also be inhibited directly by raising antibodies that bind essential structural elements like mycelial cell wall-bound proteins in *Neotyphodium coenophialum* and *F. asiaticum* (Hiatt III et al., 2001; Hu et al., 2008)

2.1.4.1. Neutralizing antibodies

Serotherapy for neutralization of toxins was first reported in 1894 and is still the treatment of choice for venoms of scorpion stings and snake bites (Phisalix and Bertrand, 1894). The traditional practice of producing antivenom immunoglobulins from immunized animals is giving way to the newer, more effective practice of engineering antivenoms from recombinant antibodies such as scFv and V\(_{H}\)H\(_{H}\) and V\(_{H}\)H\(_{2}\)-Fc fusion antivenoms exhibit higher stability, greater tissue penetration and decreased risk of an immunogenic response in a patient (Richard et al., 2013). Monoclonal antibodies can also act as effective neutralizing agents of toxins such as *Clostridium botulinum* neurotoxin type A or the shiga toxins produced by *E. coli* O157:H7 (Wu et al., 2001; Tzipori et al., 2004)

Neutralizing antibodies (nAb) provide the main type of immunity resulting from vaccination (Dimmock, 1984). Neutralization is considered to be the most efficient mode of antibody-mediated defence against viral pathogens (Huber and Trkola, 2007). As such, nAb can be utilized in the treatment and prevention of viral infection. High affinity human antiviral antibodies can be engineered to bind viral coat proteins and disrupt the viral replication cycle in the host (Maynard and Georgiou, 2000). The efficacy of
neutralization has been studied for viruses such as the hepatitis C virus, influenza virus type A and poliovirus (Zeisel et al., 2007; Dimmock, 1984; Wetz, 1993). Currently, the most frequently studied application of nAb is the development of antibodies to combat the human type 1 immunodeficiency virus (HIV-1). To date, the effects of HIV-1-targeted nAb in vivo have been, unfortunately, minute. HIV-1 is remarkably refractive to neutralization as the virus possesses several means of eluding recognition by antibodies, including masking of surface epitopes, heavy glycosylation of envelope proteins and a high mutation rate allowing rapid evolution under selection pressure. However, constant selection pressure from neutralizing antibodies could eventually restrict viral fitness and aid long-term control (Huber and Trkola, 2007).

Although largely unexplored, neutralizing antibodies could presumably be utilized to combat plant viruses as well. Additionally, neutralizing antibodies could potentially mitigate the effects of bacterial and fungal-derived plant diseases by binding the toxins and/or elicitors produced by these pathogens to prevent disease symptoms (Peschen et al., 2004). Expression of anti-plant pathogen antibodies could be achieved in planta to provide an alternative method for treatment of parasitic plant diseases (Brar and Bhattacharyya, 2012).

2.2. Pythium aphanidermatum

2.2.1. Organism characteristics

Pythium aphanidermatum is a detrimental plant pathogen capable of infecting a broad range of hosts around the world (van der Plaats-Niterink, 1981). It is a member of a parasitic group of filamentous, fungal-like microorganisms called the Oomycetes
(Hardham, 1992). Although referred to as fungi or water moulds, the Oomycetes are more closely related to the biflagellate heterokont algae (e.g. Chrysophyceae, Phaeophyceae and Xanthophyceae) of the kingdom Chromista. Thus they are classified separately from the kingdom Fungi which includes the higher fungi (namely the Zygomycetes, Ascomycetes and Basidiomycetes) (Cooke et al., 2000; Dick, 1997).

A key difference between Oomycota and higher fungi are their hyphal cell walls containing cellulose instead of chitin (Hendrix, 1975). The hyphae of *P. aphanidermatum* are colourless, transparent and up to 10 µm wide. Hyphae or mycelia form the main body of the organism and can differentiate into specialized reproductive structures such as sporangia, oogonia or antheridia. Sporangia are terminal complexes of swollen hyphae capable of forming asexual zoospores for propagation and proliferation (van der Plaats-Niterink, 1981). *P. aphanidermatum* zoospores are 12 µm in diameter upon encystment and are characterized by a posterior whiplash flagellum and an anterior tinsel flagellum which enable them to move through aquatic environments (Hardham, 1992).

*P. aphanidermatum* is also capable of sexual reproduction through the use of ‘female’ oogonia and ‘male’ antheridia reproductive structures (see Figure 2.4). Oogonia are terminal hyphal structures, while antheridia are usually intercalary and may or may not originate from the oogonial stalk (van der Plaats-Niterink, 1981). Both structures undergo meiosis before the antheridia fertilizes the oogonium to produce a diploid oospore (Sansome, 1963; Sansome, 1966). *P. aphanidermatum* oospores range from 18 to 22 µm in diameter and are aplerotic with cell walls 1 to 2 µm thick (van der Plaats-Niterink, 1981).
2.2.2. Associated disease

*Pythium aphanidermatum* primarily colonizes the roots of susceptible plants both internally and externally to cause disease symptoms such as root browning, root rot, root stunting, root hair inhibition, root swelling and tissue maceration (Owen-Going, 2002).
Shimada et al. (1999) report withering of bentgrass due to indole-3-acetic acid produced by *P. aphanidermatum*. Root browning is generally a result of the host plant producing defensive phenols and tannins against the pathogen (Stanghellini and Rasmussen, 1994). Tissue maceration is a result of the pathogen’s pectinolytic and cellulolytic capabilities. The pathogen preferentially attacks young or watery plant tissue over older or woodier tissues with secondary wall thickenings; it can also attack germinating seeds in soil to cause seedling rot (van der Plaats-Niterink, 1981; Okubara and Paulitz, 2005). Severe cases of infection can lead to stunting of the plant, wilting, stem and fruit rot, and even death of entire crops (Bates and Stanghellini, 1984). Symptomless disease development has also been reported (Blancard et al., 1992; Rafin and Tirilly, 1995; Feng and Dernoeden, 1999). *P. aphanidermatum*-mediated disease is quite common among turfgrasses, often causing root and crown rot, blight, and chlorosis, although the pathogen can be symptomless in these instances as well (Abad et al., 1994; Feng and Dernoeden, 1999). Infection has been reported in many other plants such as tomato, cucumber, peppers, flax, sugar beet, sugarcane, tobacco, alfalfa and soy, causing diseases such as root rot, damping-off, stalk and rhizome rot, soft rot, fruit rot or cottony blight (van der Plaats-Niterink, 1981).

2.2.3. Occurrence and prevalence of infection

*Pythium* species are found all over the world in both soil and aquatic environments, occurring most abundantly in the superficial layers of cultivated soils in a variety of temperature ranges. Due to its ubiquity, the genus *Pythium* has been dubbed the ‘common cold’ of crop plants (Cook and Veseth, 1991). *P. aphanidermatum* can be
found in temperate to tropical soils worldwide; it has been reported in Canada and the USA as well as many countries in South America, Britain and continental Europe, Asia, Africa, Australia and New Zealand. It grows over a temperature range of 10 to 40°C with an optimal growth range of 30 to 35°C; infection at temperatures lower than 20°C is negligible (van der Plaats-Niterink, 1981; Bates and Stanghellini, 1984; Gold and Stanghellini, 1985).

*P. aphanidermatum* infections are especially prevalent in hydroponic systems. Together, the genera *Pythium*, *Phytophthora*, and *Fusarium* cause 80% of epidemics in hydroponic systems (Schuerger, 1998). *P. aphanidermatum* is estimated to be the most common *Pythium* species found in Ontario (Zheng et al., 2000). The high prevalence of zoosporic fungi in hydroponic crops can be attributed to the growth conditions present in these systems and how they differ from those in field crops. Temperature is constant over long periods of time in a hydroponic culture; if the temperature of the nutrient solution coincides with the growth range of a pathogen then the pathogen is being provided with an environment conducive for sustained root infection. A pathogen growing in its optimal temperature range may also produce more zoospores (Stanghellini and Rasmussen, 1994). Zoospores are produced in aquatic conditions as this allows them to disperse and infect susceptible hosts (Okubara and Paulitz, 2005). Thus, the abundance of free water in hydroponic systems favours the propagation of zoosporic fungi. In addition to self-dispersal via zoosporic motility, pathogens can also spread rapidly in the culture medium via the recirculating nutrient solution or by root-to-root contact between plants (Stanghellini and Rasmussen, 1994). The ionic nature of a nutrient solution also promotes growth of fungal pathogens by allowing for easier nutrient uptake. Finally, a lack of
antagonism from microorganisms normally found in the pathogen’s ecological niche allows *P. aphanidermatum* to quickly establish and prosper (Cook and Baker, 1983; Paulitz 1997). The culminating result of these factors is that a small quantity of contamination can cause substantial loss due to disease (Paulitz, 1997). For example, Menzies et al. (1996) report that an inoculum density of $2 \times 10^5$ CFU/100 L (2 CFU/mL) can result in 75% loss of crop yield.

*P. aphanidermatum* and other pathogenic fungi can be introduced to hydroponic crops through a variety of sources such as commercial peat or washed river sand, irrigation water from rivers or streams, and inadvertent contamination with soil (Kim et al., 1975; Favrin et al. 1988; Stanghellini and Rasmussen, 1994). Common greenhouse insects such as fungus gnats (*Bradysia impatiens*) and shore flies (*Scatella stagnalis*) are also known to spread *P. aphanidermatum* among susceptible hosts (Goldberg and Stanghellini, 1990; Jarvis et al., 1993).

### 2.2.4. Mechanism of action

*P. aphanidermatum* uses zoospores to propagate in hydroponic systems. Zoospores are readily formed in the presence of free water and after a temperature drop of 2-5°C from the optimal temperature range of 25-30°C (Stanghellini, 1975; Rahimian and Banihashemi, 1979; van der Plaats-Niterink, 1981). Zoospores detect root exudates such as simple sugars, amino acids, organic acids and fatty acids and use chemotaxis to locate plant roots. They are most often attracted to growing parts of the root such as the area just behind the root tip, root hairs, or the area from which a secondary root emerges (Okubara and Paulitz, 2005). They are also attracted to wound sites on plant roots. When
the zoospore reaches the plant root it begins to encyst: the cell sheds its flagella, forms a cell wall, organelles are redistributed in the cytoplasm and an adhesive cell coat is secreted to facilitate attachment to the root surface (Hardham, 1992; Estrada-Garcia, 1990). It is thought that encystment is triggered by larger molecules closely associated with the root surface, such as pectin, as few molecules that act as attractants are also able to induce encystment (Hardham, 1992). After encystment the spore initiates germination through the production of a germ tube which differentiates into an appressorium (Okubara and Paulitz, 2005). The appressorium forms an infection peg, which facilitates penetration of the root surface through or between intact epidermal cells (Endo and Colt, 1975). Once penetration has been achieved, *P. aphanidermatum* begins colonization by growing intracellularly in the root, killing the root tissue. Hyphae may differentiate into sporangia and produce zoospores to infect new hosts. Pathogen biomass can accumulate and spread throughout the root structure until the plant dies, at which point the hyphae will differentiate into oosporic survival structures (Okubara and Paulitz, 2005). *P. aphanidermatum* is a particularly aggressive necrotroph and can cause host death within several days of infection (Menzies et al., 1995).

One of the key determinants of *P. aphanidermatum* virulence is a toxin protein produced by the organism known as NLP_{Pa} (formerly known as *Pythium aphanidermatum* Necrosis inducing elicitor, or PaNie) (Veit et al., 2001; Gijzen and Nürenberger, 2006). In 1998, Koch et al. purified a crude preparation from *P. aphanidermatum* that was able to induce several defence-associated responses in carrot cells, including a transient induction of phenylalanine ammonium-lyase (PAL), production of 4-hydroxybenzoic acid (4-HBA) and initiation of the hypersensitive
response (HR). These responses, along with the observation of DNA fragmentation (the hallmark of programmed cell death) indicated that treatment with the protein induced both the production of phytoalexins and rapid cell death (Koch et al., 1998). Using cDNA isolated from *P. aphanidermatum*, Veit et al. (2001) were able to purify the protein. The effects of purified NLP\textsubscript{Pya} on carrot cell cultures are nearly identical to the effects Koch et al. (1998) observed with the crude protein preparation: 4-HBA is produced, DNA fragmentation occurs and rapid cell death is observed. These results indicated that a single protein toxin (NLP\textsubscript{Pya}) was sufficient to trigger phytoalexin synthesis and programmed cell death (Veit et al., 2001). Injections of plant foliar tissue with *P. aphanidermatum* exudates and anti-NLP\textsubscript{Pya} antibodies have indicated that NLP\textsubscript{Pya} is a major determinant of phytotoxicity and immune activation (Fjällman, 2008).

### 2.2.5. Current methods of control

Common strategies for detection of *P. aphanidermatum* include baiting, culture plating and membrane filtration (Wakeham et al., 1997). Control methods for the pathogen are generally preventative rather than amelioratory (Tu, 2004). *Pythium* infections can be treated chemically; however, there are only two chemical fungicides currently registered for use on *Pythium* species in Canada, propamocarb and metalaxyl, to which there is a high risk of *P. aphanidermatum* developing resistance (Bitterlich et al., 2007). The nutrient solution circulated in hydroponic systems can be treated with UV radiation, filtration or ozone to eliminate potential contamination (Stanghellini et al., 1984; Goldberg et al., 1992; Runia, 1994a; Runia 1994b). While effective, these solutions are not readily applicable to large-scale systems requiring nutrient solution flow rates
exceeding 1000 L/minute (Stanghellini and Rasmussen, 1994). Amendment of nutrient solution with non-ionic surfactants or biocides like chitosan or silicates can prevent the spread of infection by eliminating zoospores before they reach their hosts (Stanghellini et al., 1996; El Ghaouth et al., 1994; Bélanger et al., 1995).

More recently, biological control has been investigated as an option for management of *P. aphanidermatum* infection. Disease in hydroponically grown plants was reduced by antagonism of the pathogen with non-pathogenic species of fungi or bacteria such as *Trichoderma* spp. or *Pseudomonas* spp., respectively (van der Plaats-Niterink, 1981; Liu et al., 2007). Indeed, the genus *Pythium* is much less prevalent in soil crops than in hydroponic crops partly because it cannot readily compete with other soil microorganisms (Paulitz, 1997). Mycoparasitic species of *Pythium* such as *P. periplocum* also have the ability to control *P. aphanidermatum* infection (Hockenhull et al., 1992).

The commensal bacterium *Pseudomonas chlororaphis* has been shown to be a very effective biocontrol agent of *P. aphanidermatum* in hydroponic plant systems (Chen et al., 2000; Liu et al. 2002). This method is not without its limitations, as effective control of *P. aphanidermatum* by *P. chlororaphis* is dependent on pre-inoculation with the biocontrol agent. By inoculating susceptible plants with *P. chlororaphis* Tx-1 four days prior to and three days following pathogen exposure, or three days following pathogen exposure only, plant root browning symptoms were significantly reduced. Pathogen incidence in plants pre-inoculated with *P. chlororaphis* was 11%, while pathogen incidence in plants inoculated with *P. chlororaphis* after pathogen exposure was 33% and in plants without *P. chlororaphis* inoculation was 62% (Khan et al., 2003). High-density application and maintenance of the agent (10^5 CFU/g fresh roots) are also
essential to limit the spread of pathogen, indicating colonization of the roots by *P. chlororaphis* is an essential step in controlling *P. aphanidermatum* infection (Chatterton et al., 2004; Chin-A-Woeng, 2000).

*P. chlororaphis* likely antagonizes *P. aphanidermatum* through a combination of competition and antibiosis. Populations of *P. chlororaphis* may reduce *P. aphanidermatum* root colonization through competition for space in the rhizosphere, especially at infection sites (Khan et al., 2003). Suppression of root colonization could also occur by *P. chlororaphis* utilizing root exudates to the extent that attraction and germination of *P. aphanidermatum* zoospores is reduced (Zhou and Paulitz, 1993). *P. chlororaphis* is known to produce the antibiotic phenazine in the rhizosphere, which effectively controls a number of root pathogens and could be a factor in *P. aphanidermatum* suppression (Pierson III and Pierson, 1996; O’Sullivan and O’Gara, 1992). Colonization of *P. chlororaphis* also induces a number of defence responses in the plant host such as phenylalanine ammonium-lyase (PAL) synthesis, increases in peroxidase (PO) and polyphenol oxidase (PPO) activity, and stimulation of the release of many other defensive phenolics, all of which may play a role in the suppression of *P. aphanidermatum* infection (Spencer et al., 2003; Nakkeeran et al., 2006).

### 2.3. Necrosis and ethylene inducing peptide 1 (Nep1)-Like Proteins (NLPs)

#### 2.3.1. Protein characteristics

NLP<sub>Pya</sub> belongs to an emerging group of proteins known as the Nep1-like proteins or NLP, named for their similarity to the necrosis and ethylene inducing peptide 1 (NLP<sub>Fo</sub>, formerly Nep1) produced by *Fusarium oxysporum* f. sp. *erthyroxyli* (Gijzen and
Nürnberger, 2006; Bailey, 1995). NLPs are 25-27 kDa proteins characterized by their ability to trigger plant defence responses, necrosis, and cell death in dicotyledonous plants. They have no known effect on monocotyledonous plants (Bailey et al., 1995; Veit et al., 2001; Fellbrich et al., 2002; Qutob et al., 2006; Schouten et al., 2008).

Most NLPs share a structural feature known as the NPP1 domain consisting of two or four cysteine residues and a central conserved heptapeptide motif ‘GHRHDWE’ (Fellbrich et al., 2002; Gijzen and Nürnberger, 2006). Many other residues show a high degree of conservation in the NLP proteins, and most NLPs possess a cleavable N-terminal sequence that targets these proteins for secretion from the originating cell (Gijzen and Nürnberg, 2006). After elucidating the structure of NLP$_{Pya}$ through x-ray crystallography, it was compared to the modelled structures of NLPs from Pectinobacterium carotovorum subsp. carotovorum and Phytophthora parasitica. Despite differences in their polypeptide sequences, these NLPs were found to match the structure of NLP$_{Pya}$ on the tertiary level, and the positions of residues in the heptapeptide ‘GHRHDWE’ motif are conserved. This indicates that these proteins are structurally and functionally homologous (Ottmann et al., 2009).

2.3.1.1. Associated symptoms

Plant cells react to NLPs with a variety of defence responses and signal cascades beginning as early as four hours post exposure. NLPs induce accumulation of calcium and reactive oxygen species (ROS) and production of ethylene (Koch et al., 1998; Fellbrich et al., 2002; Bailey, 1995). The phenylpropanoid pathway is activated, as indicated by maximal transcription of phenyl-ammonia lyase (PAL) and an increase in production of ethylene, phytoalexins and phenols (Koch et al., 1998). An increase in
salicylic acid signalling and mitogen-activated protein (MAP) kinase activity also occurs (Fellbrich et al., 2002). Changes in K⁺ and H⁺ channel fluxes and loss of membrane integrity are also observed (Jennings et al., 2000). These symptoms all culminate in programmed cell death, as shown by DNA fragmentation (Veit et al., 2001).

2.3.2. Occurrence and prevalence

NLPs have been identified in prokaryotic and eukaryotic organisms, including species of fungi, oomycetes and bacteria. Most species possessing NLPs are hemibiotrophic, necrotrophic or saprotrophic plant pathogens. The increasing availability of fully sequenced microorganism genomes has enabled identification of putative NLP coding sequences through their similarity to known NLP genes (Qutob et al., 2006; Dong et al., 2012). As such, there are many NLPs and NLP-encoding genes that have been identified but not studied. All identified NLP species that have been studied in a laboratory setting are listed in Table 2.2, along with a proposed naming scheme.

NLPs can be classified as ‘Type I’ or ‘Type II’ depending whether they possess one or both cysteine pairs in the NPP1 domain. NLPs with Type I domains are found in fungi, oomycetes and Gram positive bacteria, while NLPs with Type II domains are present only in fungi and Gram negative bacteria. Representative members of both Types can be found within a single (fungal) organism. For example, the rice blast fungus *Magnaporthe oryzae* has 4 open reading frames (ORFs) encoding NLPs – 2 of each Type (Gijzen and Nürnberger, 2006).
Table 2.2. Select NLPs, originating organism species and classification, and characteristics. ‘Proposed’ refers to a proposed NLP naming scheme based on the abbreviations introduced by Gijzen and Nürnberger (2006). ‘Expressed’ refers to whether the NLP is expressed during organism infection of a host plant and ‘Necrosis’ refers to ability to induce necrosis in susceptible plants, as indicated by a “+” or “-”. UNK indicates information is currently unknown. Information compiled from the following sources: Schouten et al., 2008; Cuesta-Arenas et al., 2010; Staats et al. 2007a; Baxter et al., 2010; Cabral et al., 2012; Zhang et al. 2012; Liu et al. 2010; Garcia et al., 2007; Zaparoli et al., 2011; Motteram et al., 2009; Mattinen et al., 2004; Feng and Li 2013; Kanneganti et al., 2006; Fellbrich et al., 2002; Qutob et al., 2006; Dong et al., 2012; Veit et al., 2001; Bashi et al., 2010; Wang et al. 2004; Santhanam et al. 2013

<table>
<thead>
<tr>
<th>Species</th>
<th>Organism</th>
<th>Name</th>
<th>Proposed</th>
<th>Type</th>
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<tr>
<td><em>Bacillus halodurans</em></td>
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<td><em>Botrytis cinerea</em></td>
<td>Ascomycete</td>
<td>BeNEP1</td>
<td>NLP_{Bc1}</td>
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<td>Ascomycete</td>
<td>BeNEP2</td>
<td>NLP_{Bc2}</td>
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<td><em>Fusarium oxysporum</em></td>
<td>Ascomycete</td>
<td>BeNLP1</td>
<td>NLP_{Bc1}</td>
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<td><em>Hyaloperonospora arabidopsis</em></td>
<td>Oomycete</td>
<td>BeNLP2</td>
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<td><em>Magnaporthe oryzae</em></td>
<td>Ascomycete</td>
<td>HaNLP1</td>
<td>NLP_{Ha1}</td>
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<td>Basidiomycete</td>
<td>HaNLP2</td>
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<td>NLP_{Mp1}</td>
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<td>-</td>
<td>+</td>
</tr>
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<td></td>
<td>MpNEP2</td>
<td>NLP_{Mp2}</td>
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### Table 2.2. Continued.

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<th>Kingdom</th>
<th>Genus</th>
<th>Gene</th>
<th>Gene Description</th>
<th>Subgroup</th>
<th>UNK</th>
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</thead>
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<tr>
<td><em>Mycosphaerella graminicola</em></td>
<td>Ascomycete</td>
<td>MgNLP</td>
<td>NLP&lt;sub&gt;Mg&lt;/sub&gt;</td>
<td>UNK</td>
<td>+</td>
<td>+</td>
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<td></td>
</tr>
<tr>
<td><em>Pectobacterium carotovora</em></td>
<td>G- Bacteria</td>
<td>Nip</td>
<td>NLP&lt;sub&gt;Pec&lt;/sub&gt;</td>
<td>II</td>
<td>+</td>
<td>+</td>
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<td></td>
</tr>
<tr>
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<td>Oomycete</td>
<td>phcnlp1</td>
<td>NLP&lt;sub&gt;pc&lt;/sub&gt;</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phytophthora infestans</em></td>
<td>Oomycete</td>
<td>PiNPP1.1</td>
<td>NLP&lt;sub&gt;pi&lt;/sub&gt;</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phytophthora parasitica</em></td>
<td>Oomycete</td>
<td>NPP1</td>
<td>NLP&lt;sub&gt;pp&lt;/sub&gt;</td>
<td>I</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Verticillium dahliae</em></td>
<td>Ascomycete</td>
<td>NLP1</td>
<td>NLP&lt;sub&gt;vd&lt;/sub&gt;</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio pommerensis</em></td>
<td>G- Bacteria</td>
<td>orf 9</td>
<td>NLP&lt;sub&gt;v&lt;/sub&gt;</td>
<td>II</td>
<td>UNK</td>
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Another unusual aspect of NLP distribution is their occurrence in host organisms that are not plant pathogens. For instance, the fungi *Aspergillus nidulans* and *Neurospora crassa* possess two and one NLP, respectively, despite neither having shown pathogenic activity on plants (Gijzen and Nürnberger, 2006). *A. nidulans*, however, is known to possess opportunistic pathogenicity in humans (Denning, 1998). Perhaps the most extreme example is the gram-negative bacterium *Vibrio pommerensis*, which possesses an NLP even though the organism was isolated from seawater and is not known to colonize plants at all (Jores et al., 2003). Many other NLP producing organisms are known pathogens of monocotyledonous plants which is again unusual since NLPs have been shown to induce effects exclusively in dicotyledonous plant cells, despite extensive testing on monocotyledonous plants. Indeed, the fungus *Mycosphaerella graminicola*, a pathogen of wheat, has been shown to express an NLP during infection of wheat despite the NLP’s lack of observable effect on the plant (Motteram et al. 2009).

2.3.2.1. Evolution within and across diverse phylogenies

The occurrence of such conserved protein structures in both prokaryotic and eukaryotic organisms is highly unusual, and likely results from a horizontal gene transfer event (Pemberton and Salmond, 2004). Comparison of the nucleotide content of NLP genes vs. the genomes in which they are located has been cited as evidence that these genes were at one time foreign. For instance, in *V. pommerensis*, its genome G+C content is 50-60% while its NLP gene G+C content is only 25-35%. This is also the case in *P. carotovora* subsp. *amylovora*; the average G+C content of its genome is 50.9%, while its NLP gene G+C content is only 42.5%. In many other cases the G+C content of an NLP
gene is close to that of the genome in which it is found. This would suggest that NLPs are ancient or longstanding genes in many organisms (Pemberton and Salmond, 2004).

The NLP gene family in oomycetes is quite expanded compared to those in fungal or bacterial phytopathogens (Dong et al., 2012; Cabral et al., 2012). Analysis of the *Phytophthora sojae* genome revealed a family of 33 NLP genes interspersed with 37 pseudogenes. Of the 33 genes, 20 were expressed during *P. sojae* infection and only eight were capable of inducing necrosis (Dong et al., 2012). Similar clusters of NLP genes and pseudogenes have also been observed in the genomes of *Phytophthora ramorum* and *Phytophthora infestans* (Haas et al., 2009; Tyler et al., 2006). A cluster of 12 genes and 15 pseudogenes was also discovered in the genome of the oomycete *Hyaloperonospora arabidopsis*. Although ten of the 12 genes were expressed during infection, none were capable of inducing necrosis. Additionally, the expressed NLPs ranged in length from 173 to 419 amino acids and the GHRHDWE motif was degenerated in all but two (Cabral et al., 2012). Feng and Li (2013) reported the discovery of an NLP gene in *Phytophthora capsici* that was 1431bp and encoded an NLP 476 amino acids long; as such, both gene and gene product were double the length of most other NLPs. Accordingly, this NLP contains two repeats of the conserved GHRHDWE motif (Feng and Li, 2013). The large NLP gene families in oomycete species, as well as the prevalence of pseudogenes and the variation among amino acid substitution ratios suggests that these genes arose from a single ancestor and are under diversifying selection pressure (Dong et al., 2012).

Horizontal gene transfer of the expanded and diversified oomycete NLP gene families could explain the occurrence of non-cytotoxic NLPs in other plant pathogens. It is possible that these genes or pseudogenes were part of a horizontal transfer with other
functional genes to a different organism or pathogen. Alternatively, these genes could have mutated (as they likely did in *Phytophthora*) from functional NLPs to proteins that no longer induce necrosis (Gijzen and Nürnberg, 2006). *Phytophthora* species such as *P. sojae* and *P. infestans* express their NLPs as they transition from biotrophy to necrotrophy, suggesting that these cytotoxic NLPs play a role in the transition (Qutob et al., 2002; Kanneganti et al., 2006). Similarly, non-cytotoxic NLPs may play another, as-yet-unidentified, role in the establishment of biotrophic interactions by oomycetes like *H. arabidopsis* (Cabral et al., 2012).

2.3.3. Proposed mechanism of action

Although some NLPs have been found to induce plant defences, necrosis and cell death, the exact biological function of these proteins is still being elucidated. The fungus *Colletotrichum coccodes* showed a marked increase in virulence when transformed with NLP<sub>Fo</sub>, as well as a much broader host range (Amsellem et al., 2002). Strains of *P. carotovora* lacking NLP<sub>Pcc</sub> cause far fewer lesions and less rot of tissues than strains expressing the protein (Mattinen et al., 2004). *P. sojae* expresses NLP<sub>Psa1</sub> during its transition from biotrophy to necrotrophy which indicates that this protein may aid in host colonization by accelerating plant cell death (Qutob et al., 2002).

NLPs are thought to cause necrosis by forming pores in the membranes of plant cells. This theory was initially proposed after NLP<sub>Bc1</sub> and NLP<sub>Bc2</sub> were shown to lyse protoplasts and release dye contained within. These NLPs were also found to associate with membranes and nuclei of plant cells (Schouten et al., 2008). Subsequent vessel leakage assays with NLP<sub>Pya</sub> have demonstrated its ability to cause disintegration of
membrane vesicles from *Arabidopsis thaliana* and *N. tabacum* but not from *Commelina communis*, which is consistent with the observed lack of NLP activity in monocots (Ottmann et al., 2009).

Crystal structure analysis of NLP<sub>Pya</sub> revealed its resemblance to actinoporins from sea anemones *Actinia equina* and *Stychodactyla helianthus* and fungal lectins from *Agaricus bisporus* and *Xerocomus chrysenteron*. Like NLPs, actinoporins and lectins are proteins that target lipid bilayers and are small, single-domain polypeptides with β-sandwich architecture joined by broad loops. Actinoporins act cytolytically by forming transmembrane pores via insertion of their N-terminal regions into the membrane. This is especially interesting, since it has already been shown that NLP activity is destroyed by any deletion in the N-terminus. Thus, a possible model for NLP membrane attachment is for it to use its hydrophobic N-terminus to penetrate a lipid membrane, and possibly mediate this through the binding of a Ca<sup>2+</sup> at the membrane surface (Küfner et al., 2009).

### 2.3.3.1. Functional significance of NPP1 domain

Using x-ray crystallographic data of NLP<sub>Pya</sub>, formation of an intramolecular disulfide bridge between the two conserved cysteine residues has proved to be essential for protein activity. The crystal structure also revealed a cavity exhibiting a strong negative charge formed by residues D93, D104, E106 and H159 and stabilized by H101 (corresponding to residues GHRHIDWE in the conserved heptapeptide motif). Experiments with site-directed mutagenesis confirmed that these residues are essential for NLP<sub>Pya</sub> to induce necrosis in *N. tabacum* leaves, lyse *N. tabacum* plasma membranes and complement virulence in *NLP<sub>Pce</sub>* (i.e., *P. carotovora* subsp. *carotovora* mutants). The negatively charged cavity was shown to bind a cation; based on its abundance at the
extracellular membrane surface, this cation could be Ca\(^{2+}\). NLP activity is abolished by the calcium chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetracetic acid (BAPTA), which suggests that coordination of a Ca\(^{2+}\) cation in the cavity is also crucial for NLP function (Ottmann et al., 2009).

![Crystal structure of NLP\(_{Pya}\)](image)

**Figure 2.5. Crystal structure of NLP\(_{Pya}\).** A, Ribbon plot of NLP\(_{Pya}\); secondary structure elements are shown in blue (\(\alpha\)-helices) and green (\(\beta\)-strands); a bound magnesium atom is shown as a blue sphere. B, Electron density for the bound magnesium and its coordinating residues in NLP\(_{Pya}\); the 2F\(_{o}\) - F\(_{c}\) density map (contoured at 1 \(\sigma\)) is shown in black. C, Comparison of NLP\(_{Pya}\) (orange) with modeled structures of NLP\(_{Pp}\) (blue) and NLP\(_{Pcc}\) (green). Residues involved in ion binding (sticks in B and C) or formation of the disulfide bond (sticks in A and C) are labeled. NGA, N-acetylgalactosamine; POC, phosphorylcholine. Diagram modified from Ottmann et al., 2009.

2.3.3.2. Classification as virulence factors

Plant immunity is triggered by the detection of pathogen-associated molecular patterns (PAMPs) using pattern recognition receptors, or by pathogen-specific effector molecules like virulence factors (Nürnberger and Kemmerling, 2006). When NLPs were first discovered they were referred to as ‘elicitors’, which implied that NLPs are PAMPs.
However, NLPs differ from known PAMPs in several respects: they are transiently expressed proteins, rather than constitutively expressed structural features like flagellin; an elicitor-active minimal motif (such as flg-22 from flagellin) has not yet been identified; cell death induced by NLPs differs from cell death in hypersensitive response (HR); and finally, PAMPs rarely trigger HR cell death in plants, yet the necrosis induced by NLPs is a constant and defining feature of these proteins (Thomma et al., 2011; Küfner et al., 2009).

Despite their incredibly broad host range and their occurrence in non-hosts and non-phytopathogenic organisms, the characteristics of NLPs suggest they are virulence factors and toxins. It has been shown that strains of *P. carotovora* subsp. *carotovora* lacking a functional NLP gene (*NLP_Pcc*) also show a corresponding lack of virulence in plants. Virulence of these *NLP_Pcc* mutants can be restored by the addition of genes encoding *NLP_Pya* or *NLP_Pp* (Ottmann et al., 2009). More recent publications have demonstrated that *NLP_Pya* (and therefore other NLPs) has cell-lysing activity and is likely a pore-forming toxin (Küfner et al., 2009). Thus, a plant’s immune reaction to an NLP (through the production of ethylene, etc.) may be due to membrane disruption and cell death; i.e. the plant is reacting to the action of the NLP and not the protein itself. The evidence suggests that many NLPs act as virulence factors by killing plant cells to aid pathogen growth in host tissues (Gijzen and Nürnberg, 2006).
3. **Development of a Cross-Reactive Polyclonal Antibody Assay for Detection of Nep1-Like Proteins**

3.1. **Introduction**

In 2001, a protein named PaNie (*Pythium aphanidermatum* Necrosis-inducing elicitor) was identified as the elicitor responsible for induction of a hypersensitive-like response and necrosis in *A. thaliana*, *N. tabacum* and *Lycopersicon esculentum* (tomato) plants during infection by *Pythium aphanidermatum*. Its 234 amino acid sequence was published and it was noted that the protein’s lethal effects were limited to dicotyledenous plant cells only (Veit et al., 2001). Now identified as NLP$_{Pya}$, this protein is part of a growing family of proteinaceous elicitors known as the Nep1-Like Proteins, or NLP. NLPs obtained their name after the first Nep1 (Necrosis and ethylene inducing peptide 1, now NLP$_{Fo}$) from *Fusarium oxysporum* f. sp. *erythoxyli*. This group of proteins can be easily identified by a conserved heptapeptide motif “GHRHDWE” (the NLP-heptapeptide) and one or two pairs of cysteine residues. NLPs have been identified in both prokaryotic and eukaryotic organisms, including bacteria, oomycetes, and fungi (Gijzen and Nürnberger, 2006). They are prolific among *Phytophthora* species and are the key factor in pathogenesis for many plant pathogens, including *Pythium aphanidermatum* (Dong et al., 2012; Ottmann et al., 2009).

NLP$_{Pya}$ has been the source of much scientific interest due to the deleterious effect *Pythium aphanidermatum* has on plants, and its role in producing plant disease. In 2009, Ottmann et al. published the crystal structure of NLP$_{Pya}$. Based on the resemblance of NLP$_{Pya}$ to actinoporins and the ability of NLPs to lyse plant cells, it was theorized that NLPs cause necrosis in plant cells by forming pores in the cell membranes. The
conserved NLP-heptapeptide motif was found to be part of a negatively charged cavity on the protein’s surface that coordinated a divalent cation; it was also found that NLP$_{Py}$ was rendered non-functional without the entire “GHRHDWE” motif intact (Ottmann et al., 2009). Given its conservation among all NLPs, the NLP-heptapeptide appeared to be a key structural motif for the functionality of this family of proteinaceous virulence factors.

NLP$_{Py}$ was chosen as a target for study based on its role in pathogenesis and the prevalence of *Pythium aphanidermatum*-induced disease in Ontario greenhouse crops (Sopher and Sutton, 2011). Previously, in the Hall laboratory, NLP$_{Py}$ was cloned from *Pythium aphanidermatum* genomic DNA for expression in *E. coli*. Using recombinant NLP$_{Py}$, rabbits were immunized and their serum harvested; the serum was shown to have a high anti-NLP$_{Py}$ titre (Fjällman, 2008). The purpose of this chapter was to establish whether this serum, raised against one specific NLP, could be used to bind and detect other NLPs based on their conserved NLP-heptapeptide motif. In order to test this theory, three additional NLPs were cloned for expression and study: the archetype NLP$_{Fo}$ from *Fusarium oxysporum* f. sp. *erythroxyl*, the unstudied NLP$_{Blu}$ from *Bacillus halodurans* and the hypothetical NLP$_{Ssc}$ from *Sclerotinia sclerotiorum* (cloned from an unexpressed gene sequence discovered in the fungus’ genome). The rabbit anti-NLP$_{Py}$ serum was investigated in immunoblot and ELISA formats to determine if it would bind NLP$_{Py}$ and the three other NLPs. The result is an assay that could detect four of these NLPs in nM quantities.
3.2. Methods and Materials

3.2.1. Cloning of recombinant NLPs

Samples and sequences of genes for NLP\textsubscript{Bha}, NLP\textsubscript{Fo}, and NLP\textsubscript{Ssc1} were provided by the Gijzen lab (Agriculture and Agri-Food Canada, London, ON). NcoI and XhoI restriction sites were added to the NLP\textsubscript{Bha} and NLP\textsubscript{Fo} genes by PCR (see Table 3.1 for primers used). An MscI site was added to NLP\textsubscript{Ssc1} in the place of an NcoI site to avoid addition of extraneous codons; an XhoI site was also added (see Table 3.1). NLP genes were amplified by PCR using the following reaction mixture: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\textsubscript{2}, 5 U Taq DNA polymerase (New England Biolabs, Ipswich, MA), 200 \(\mu\)M dNTPs (Fermentas, Ottawa, ON), 200 nM each of forward and reverse primers, and 1 \(\mu\)g source DNA, in a 50 \(\mu\)L reaction. The thermal cycle used for amplification was: 94°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min; 72°C for 5 min. Amplicons were ligated into the pCR2.1-TOPO vector using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), transformed into \textit{E. coli} XL1-Blue cells via electroporation (25 \(\mu\)F, 200 \(\Omega\), 1800 V in a 1 mm cuvette), and plated on LB agar containing carbenicillin and X-gal (75 and 80 \(\mu\)g/mL, respectively). Using the NLP gene-specific primers, white colonies were screened for the presence of NLP genes via colony PCR. Positive clones were grown overnight at 37°C in 10 mL LB-carb cultures (75 \(\mu\)g/mL) and plasmids were harvested using a Miniprep kit (Qiagen, Mississauga, ON). pCR2.1-TOPO plasmids containing NLP genes and pET28b+ plasmids were double digested with appropriate restriction enzymes (NcoI/XhoI for NLP\textsubscript{Bha} and NLP\textsubscript{Fo}; MscI/XhoI for NLP\textsubscript{Ssc1}), run on a 1% agarose gel, then extracted and purified using a QIAquick gel extraction kit (Qiagen, Mississauga, ON) to isolate NLP gene fragments.
and fully digested pET28b+ vector. Samples of vector and each gene were run on another agarose gel and quantified using a Gel Doc system and software (Bio-Rad, Mississauga, ON). Ligation mixtures of 10 µL were established using a 1:1 stoichiometric ratio of gene to vector, plus 1 U T4 ligase in supplied buffer (Fermentas, Ottawa, ON); mixtures were ligated overnight at 16°C, transformed into *E. coli* DH5α cells via heat shock and plated on LB agar plus carbenicillin (75 µg/mL; LB-carb). Using NLP gene-specific primers, colonies were screened for the presence of NLP genes via colony PCR. Positive transformants were grown overnight at 37°C in 10 mL LB-carb cultures (75 µg/mL) and plasmids were harvested using a Miniprep kit (Qiagen, Mississauga, ON). Finally, pET28b+ vectors, each containing a specific NLP gene, were transformed into *E. coli* BL21 (DE3) cells by electroporation (25 µF, 200 Ω, 2500 V in a 1 mm cuvette). Transformants were once again screened by colony PCR and gene integrity was verified by DNA sequencing.

**Table 3.1. Primers for NLP cloning.** Primers were designed for cloning NLPs Bha, Fo and Sscl into expression vector pET28b+. Each primer sequence was preceded by several thymidine repeats (red text) to facilitate proper replication. Restriction sites (capital letters) were added: NcoI (blue text) to forward primers Bha and Fo; MscI (purple text) to forward primer Sscl; XhoI (green text) to reverse primers. Start codons (ATG) are noted in bolded text.

<table>
<thead>
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<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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</tr>
<tr>
<td>BhaRev</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
</tbody>
</table>
3.2.2. Expression and purification of NLPs in *E. coli* BL21 (DE3)

NLP\textsubscript{Bha}, NLP\textsubscript{Fo} and NLP\textsubscript{Sscl} were expressed in *E. coli* BL21 (DE3) using the same conditions optimized for NLP\textsubscript{Pya}. Starter cultures of 10 mL LB plus carbenicillin and chloramphenicol (75 µg/mL and 34 µg/mL, respectively) were grown overnight at 37°C and 220 rpm. Each starter culture was added to 1 L of LB plus carbenicillin (75 µg/mL) and grown at 37°C and 220 rpm until an OD\textsubscript{600} of 0.7 was obtained. Expression was induced in large-scale cultures by the addition of IPTG to a final concentration of 1 mM and cells were grown for 4 more hours before being harvested by centrifugation (8000 x g for 10 min) and stored at -20°C.

Frozen-cell pellets were solubilized in 20 mL denaturing IMAC buffer (10 mM HEPES plus 500 mM NaCl and 8 M urea, pH 7) and sonicated on ice for 6 pulses of 30s using a Model 550 Sonic Dismembranator (Fisher Scientific, Ottawa, ON). Cell lysate was centrifuged to separate soluble and insoluble cell fractions. Soluble cell fraction was loaded on a 5 mL Hi-Trap Ni\textsubscript{NTA} column and purified using an ÄKTA FPLC system (GE Healthcare Bio-sciences, Baie d’Urfe, QC). The column was equilibrated using denaturing IMAC buffer prior to sample loading, and washed with more denaturing IMAC buffer after the sample was loaded. Elution was performed using a stepwise gradient of imidazole in denaturing IMAC buffer (10 mM HEPES plus 500 mM NaCl, 8 M urea and 500 mM imidazole, pH 7); imidazole concentrations used were 25 mM, 50 mM, 62.5 mM, 75 mM, 100 mM, 150 mM, 200 mM, 250 mM and 500 mM. Eluted fractions were analysed by SDS-PAGE and fractions containing NLP were desalted and passively renatured by dialysis in PBS at 4°C. Dialysed samples were concentrated in dialysis tubing using PEG-35000. An additional purification step was performed using a
Superdex 75 10/300 size exclusion column on FPLC (GE Healthcare Bio-sciences, Baie d’Urfe, QC). NLP concentrations were determined by measuring their absorbance at 280 nm (see Table 3.2).

3.2.3. Immunization of rabbits and serum preparation

Previously, three NZW outbred rabbits (female, 2.5-3.0 kg, 11-13 weeks old) were injected subcutaneously with 25 µg NLP<sub>Pya</sub> in a 1:1 PBS:Titremax solution (Sigma-Aldrich, Oakville, ON). Rabbits were injected every two weeks with 250 µL, for a total of four injections over nine weeks; the final two injections were performed without Titremax. Rabbits were bled in the weeks between injections; terminal heart bleeds were performed two weeks after the last injection. Serum was prepared by centrifuging at 2700 x g for 10 min at 4°C and clotting at room temperature for 4 h (Fjällman, 2008).

3.2.4. Anti-NLP-heptapeptide FPIA

NLP-heptapeptide (GHRHDWE) was synthesized with a small linker (SGSGC) and conjugated to 5-carboxy fluorescein (5-FAM) by Immunoprecise Antibodies (Victoria, BC). Optimal concentration of 5-FAM NLP-heptapeptide tracer was determined by mixing serial dilutions of 100 µL ranging from 50 µg/mL to 59 fg/mL in a microtitre plate; the plate was incubated in the dark for 15 minutes at room temperature before being read. An optimal concentration of 3 ng/mL was determined as the lowest concentration of tracer with emission readings discernable from PBS (see Figure 3.3.A). Rabbit anti-NLP<sub>Pya</sub> serum was tested for optimal binding to 5-FAM NLP-heptapeptide by mixing serial dilutions of serum from neat to 1/8 388 608 and mixing 80 µL of each
dilution with 20 µL 3 ng/mL tracer; each plate was incubated for 15 minutes in the dark at room temperature before being read. A CI-FPIA was conducted by mixing serial dilutions of 40 µL free NLP-heptapeptide, ranging from 1 mg/mL to 120 pg/mL, with 40 µL rabbit anti-NLP<sub>Pya</sub> serum (neat); the mixture was incubated for 30 minutes at room temperature. Treatments were then mixed with 20 µL 3 ng/mL tracer and incubated for 15 minutes in the dark at room temperature before the plate was read. Plates were read by subjecting wells to an excitation wavelength of 488 nm, then measuring emission at 512 nm. All dilutions were mixed in triplicate and in final volumes of 100 µL.

3.2.5. Anti-NLP immunoblotting

Purified NLP samples (1 µg each) were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to PVDF membrane (Bio-Rad, Mississauga, ON). For immunoblot analysis, membranes were blocked in 3% skim milk (EMD Chemical, Gibbstown, NJ) overnight at room temperature. Blots were probed with rabbit anti-NLP<sub>Pya</sub> serum diluted 1/3000 in PBS, then probed with goat anti-rabbit-alkaline phosphatase (AP) secondary antibody (Pierce Biotechnology, Rockford, IL) diluted 1/6000 in PBS, and developed for five minutes. Alternatively, blots were probed with mouse anti-6xHis primary antibody (Abcam, Toronto, ON) diluted 1/2000 in PBS, then probed with goat anti-mouse-AP secondary antibody (Pierce Biotechnology, Rockford, IL) diluted 1/2500 in PBS, and then developed for ten minutes. All antibodies were incubated for one hour at room temperature with shaking. Blots were washed three times for five minutes each with phosphate-buffered saline plus 0.05% Tween-20 (PBST) between each step, and
developed with 10 mL nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) substrate (Pierce Biotechnology, Rockford, IL).

3.2.6. Anti-NLP checkerboard ELISA

Microtitre wells were coated overnight at 4°C with NLP serial dilutions ranging from 8 µM to 0.125 µM in PBS. After decanting and washing, wells were blocked with 200 µL of 1.5% casein (Thermo Fisher Scientific, Ottawa, ON) in PBS for two hours at room temperature. Rabbit anti-NLP<sub>Py</sub><sub>a</sub> serum in serial dilutions ranging from 1/300 to 1/153600 in PBS, as well as rabbit pre-immune serum (neat), were added to the wells and incubated for one hour at room temperature. Goat anti-rabbit-horseradish peroxidase (HRP) secondary antibody (Pierce Biotechnology, Rockford, IL) diluted 1/6000 in PBS was added to the wells and incubated for one hour at room temperature. Plates were developed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate, then quenched with 0.1 M H<sub>2</sub>SO<sub>4</sub> after 60 seconds, and read at 450 nm. All reagents and samples were applied in aliquots of 100 µL unless otherwise stated; plates were washed three times between each step by pipetting 300 µL PBST into each well prior to decanting.

3.2.7. Anti-NLP CI-ELISA

Microtitre wells were coated overnight at 4°C with 100 µL of 1 µM NLP in PBS. After decanting and washing, wells were blocked with 200 µL 1.5% casein in PBS for two hours at room temperature. Rabbit anti-NLP<sub>Py</sub><sub>a</sub> serum in dilutions ranging from 1/1200 to 1/20 000 in PBS was pre-incubated with serial dilutions of each NLP for one hour at room temperature in microcentrifuge tubes, then added to the wells and incubated
for one hour at room temperature. Goat anti-rabbit-HRP diluted 1/6000 in PBS was added to the wells and incubated for one hour at room temperature. Plates were developed using TMB substrate, quenched after ten minutes with 0.1 M H$_2$SO$_4$, then read at 450 nm. All reagents and samples were applied in triplicate (100 µL) unless otherwise stated; plates were washed five times between each step by pipetting 300 µL PBST into each well followed by decanting. CI-ELISAs were performed a minimum of three times for each NLP, on different days and using freshly diluted reagents.

3.3. Results

3.3.1. NLP cloning, expression and purification in *E. coli* BL21 (DE3)

The genes for NLP$_{Bha}$, NLP$_{Fo}$ and NLP$_{Ssc}$ were all successfully cloned from their starting material into the vector pET28b+ and expression strain *E. coli* BL21 (DE3). Primers for all three genes were designed from gene sequences provided by the Gijzen lab (Agriculture and Agri-Food Canada, London, ON). Signal sequences for all three proteins were not included in the final cloned sequences. Insertion into the vector pET28b+ allowed the addition of a C-terminal His-Tag to each NLP. Using the ProtParam tool online, the molecular weights and extinction coefficients of the NLPs were calculated, as well as several other protein characteristics (see Table 3.2). Protein molecular weights were confirmed using SDS-PAGE.
Table 3.2. Summary of calculated NLP characteristics. Using the online ProtParam tool and translated NLP sequences, the molecular weight (MW), theoretical isoelectric point (pI), extinction coefficient at 280 nm (ε), instability index (II) and grand average of hydropathy (GRAVY) were calculated for each NLP with a C-terminal His-Tag (Gasteiger et al., 2005).

<table>
<thead>
<tr>
<th>NLP</th>
<th>MW (Da)</th>
<th>Amino Acids</th>
<th>pI</th>
<th>ε (M⁻¹ cm⁻¹)</th>
<th>Instability Index (II)</th>
<th>GRAVY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bha</td>
<td>23819.4</td>
<td>214</td>
<td>6.6</td>
<td>61438</td>
<td>40.62 (unstable)</td>
<td>-0.47</td>
</tr>
<tr>
<td>Fo</td>
<td>25266</td>
<td>234</td>
<td>8.46</td>
<td>58963</td>
<td>37.48 (stable)</td>
<td>-0.601</td>
</tr>
<tr>
<td>Pya</td>
<td>23999.4</td>
<td>234</td>
<td>6.95</td>
<td>47700</td>
<td>34.71 (stable)</td>
<td>-0.274</td>
</tr>
<tr>
<td>Sscl</td>
<td>23981.5</td>
<td>224</td>
<td>4.88</td>
<td>48025</td>
<td>42.48 (unstable)</td>
<td>-0.296</td>
</tr>
</tbody>
</table>

Expression of NLP_{Pya} was previously optimized in *E. coli* BL21 (DE3) (Fjällman, 2008). Using these optimized conditions, NLP_{Bha}, NLP_{Fo} and NLP_{Sscl} expressed at 10-20 mg protein per L cell culture. IMAC purification using denaturing conditions (8 M urea) was successful, as was native refolding during dialysis (see Chapter 5). All four NLPs were found to elute predictably from the Ni-NTA column between 62.5 mM and 150 mM imidazole. SDS-PAGE analysis confirmed the NLPs were present and purified in these fractions. NLP_{Bha}, NLP_{Pya} and NLP_{Sscl} were all seen as a band at approximately 24 kDa, while NLP_{Fo} was seen as a band at 25 kDa. Western blot analysis further confirmed the sizes and purity of the NLPs, as well as the presence and functionality of the His-Tag as an antigenic marker for each protein (see Figure 3.1). An untagged bovine serum albumin (BSA) control appeared on the gel at its expected size (66 kDa) but not on the immunoblot, while a His-tagged V₇H appeared at its expected size (15 kDa) on both. NLP_{Sscl} also had a second, slightly larger band at approximately 25 kDa; this band appeared on both the gel and the immunoblot.
Figure 3.1. NLP expression profile in *E. coli* BL21 (DE3). NLPs were expressed in *E. coli* using an IPTG-inducible system. Cells were lysed via sonication, centrifuged to separate soluble (supernatant) and insoluble cellular material, and the supernatant was purified via IMAC under denaturing conditions (8 M urea). Purified samples were analyzed by reducing Coomassie-stained SDS-PAGE (A) and mouse anti-6xHis-AP probed immunoblot (B). Lane 1: protein molecular weight standard; Lane 2: purified NLP<sub>Bha</sub>; Lane 3: purified NLP<sub>Fo</sub>; Lane 4: purified NLP<sub>Pya</sub>; Lane 5: purified NLP<sub>SscI</sub>; Lane 6: purified anti-NLP<sub>Pya</sub> V<sub>H</sub>H, His-tagged; Lane 7: commercial BSA, untagged. All samples were loaded to approximate 1 µg per well.
After purifying NLP by IMAC, a second purification was performed using a size exclusion column. NLP_{Bha}, NLP_{Fo} and NLP_{Pya} all eluted predictably in the 10-15 mL range, typical of proteins 25 kDa (see Figure 3.2.A-C). NLP_{Sscl}, however, eluted in the 5-10 mL range, indicating the eluted proteins were much larger than 25 kDa and suggesting the presence of an aggregate (see Figure 3.2.D). This was confirmed by the presence of a second peak in the expected 10-15 mL range; the A_{280} of this second elution was very low compared to the first one (~25 mAU vs. ~175 mAU), indicating it comprised a small proportion of the sample being tested.

3.3.2. Serum titres of rabbits in response to NLP_{Pya} immunizations

Previously, rabbits were immunized with recombinant NLP_{Pya} and their sera tested in ELISA format to determine anti-NLP_{Pya} titre. A signal to noise ratio greater than four was observed when testing binding of serum to 200 nM NLP_{Pya}, at a maximum dilution of 1/102 400. There was no increase in titre after day 21, or 2\textsuperscript{nd} injection (Fjällman, 2008).

3.3.3. Rabbit anti-NLP_{Pya} serum binding to NLP-heptapeptide

Rabbit anti-NLP_{Pya} serum was tested for ability to bind the conserved NLP-heptapeptide GHRHWDE in FPIA format. NLP-heptapeptide conjugated to 5-FAM was optimized as a fluorescent tracer; optimal concentration was determined to be 3 ng/mL (see Figure 3.3). Using the optimized concentration, serial dilutions of rabbit anti-NLP_{Pya} serum were tested for binding to 5-FAM NLP-heptapeptide. An increase in signal (measured in millipolarization units, mP) was observed at the highest serum
Figure 3.2. Summary of NLP purification using size exclusion chromatography. NLPs were expressed in *E. coli* and purified by IMAC on FPLC. A second purification was performed by passing the pre-purified NLPs through a size exclusion column (on FPLC) with a molecular weight range of 3 to 70 kDa. Collected fractions were analyzed by spectrophotometric readings at 280 nm to verify peak protein concentrations. A-D, NLP proteins; E, normalized overlay of unrelated *Listeria* proteins Internalin A (51 497 Da) and Internalin B (24 795 Da) purified under same conditions.
Figure 3.3. Anti-NLP<sub>Pya</sub> rabbit serum binding to NLP-heptapeptide. A: tracer optimization; 5-FAM NLP-heptapeptide was serially diluted from 50 µg/mL to 59 fg/mL; B: antibody optimization; anti-NLP<sub>Pya</sub> rabbit serum was serially diluted from neat to 1/8 388 608 and 80 µL of each dilution was mixed with 20 µL of optimally diluted tracer; C: inhibition FPIA; NLP-heptapeptide was serially diluted from 1 mg/mL to 120 fg/mL and 40 µL of each dilution was mixed with 40 µL anti-NLP<sub>Pya</sub> rabbit serum; mixture was incubated for 30 minutes then mixed with 20 µL of optimally diluted tracer. All dilutions were mixed in triplicate, incubated minimum 15 minutes in the dark, then subjected to an excitation wavelength of 488 nm. Emission was measured at 512 nm and plotted in millipolarization units (mP). Arrows indicate optimal dilutions determined.
concentrations tested; at serum concentrations lower than 1/32, the signal was indiscernible from background (see Figure 3.3.B). A CI-FPIA was attempted with free NLP-heptapeptide as the inhibitor and using the highest serum concentration possible (neat), but no inhibition trend was observed (see Figure 3.3.C).

3.3.4. NLP immunoblotting with anti-NLP<sub>pya</sub> serum

Rabbit anti-NLP<sub>pya</sub> serum was tested for the ability to bind other NLP in immunoblot format. NLP<sub>Bha</sub>, NLP<sub>Fo</sub>, NLP<sub>Sscl</sub>, NLP<sub>pya</sub> and appropriate controls were run on SDS-PAGE in equal amounts (1 µg each) and transferred to PVDF membranes. One membrane was probed with the rabbit anti-NLP<sub>pya</sub> serum and an appropriate secondary antibody (see Figure 3.4.C). This blot showed bands at approximately 24 kDa corresponding to NLP<sub>pya</sub> (as expected), as well as NLP<sub>Bha</sub> and NLP<sub>Sscl</sub>. No band corresponding to NLP<sub>Fo</sub> was visible. Furthermore, no bands corresponding to the controls (BSA and a His-tagged V<sub>H</sub>H) were visible. A duplicate immunoblot was probed with mouse anti-6xHis antibody in tandem to the anti-NLP<sub>pya</sub> blot (see Figure 3.4.B). All four NLP showed bands at their expected sizes on this blot, as did the His-tagged V<sub>H</sub>H control. As expected, the untagged BSA control did not show a band on this immunoblot.
Figure 3.4. Western blot analysis of anti-NLP<sub>Pya</sub> rabbit serum binding to four NLPs. Purified protein samples were run (1 µg/well) on reducing SDS-PAGE gels. Gels were run in triplicate then analyzed by Coomassie stain (A), immunoblot probed with mouse anti-6xHis-AP (B), or immunoblot probed with anti-NLP<sub>Pya</sub> rabbit serum and goat anti-rabbit-AP (C). Lane 1: protein molecular weight standard; Lane 2: purified NLP<sub>Bha</sub>; Lane 3: purified NLP<sub>Fo</sub>; Lane 4: purified NLP<sub>Pya</sub>; Lane 5: purified NLP<sub>Ssc</sub>; Lane 6: purified anti-NLP<sub>Pya</sub> V<sub>H</sub>H<sub>H</sub> His-tagged; Lane 7: commercial BSA, untagged.
3.3.5. NLP detection and binding with anti-NLP\textsubscript{Pya} serum

The ability of rabbit anti-NLP\textsubscript{Pya} serum to bind various NLP concentrations was compared in checkerboard ELISA format using the same serial dilutions of serum and coated antigen for all four NLPs. All NLPs showed a consistent trend of high binding at highest NLP coat and serum concentrations (as assessed by OD\textsubscript{450}), which decreased proportionately as serum concentration decreased (see Figure 3.5). The magnitude of binding, however, differed between NLPs. NLP\textsubscript{Pya} showed the highest binding at lower serum concentrations with OD\textsubscript{450} beginning to decrease noticeably at the 1/9600 dilution, while the OD\textsubscript{450} for NLP\textsubscript{Bha}, NLP\textsubscript{Fo} and NLP\textsubscript{Sscl} all decreased noticeably after 1/300 dilution. Binding with 1/300 serum was, however, much greater in magnitude for NLP\textsubscript{Bha} and NLP\textsubscript{Sscl} (OD\textsubscript{450} between 2.0 and 3.0) when compared to NLP\textsubscript{Fo} binding (OD\textsubscript{450} \geq 1.5). NLP\textsubscript{Bha} and NLP\textsubscript{Fo} binding also decreased proportionately with NLP coat concentration, while coat concentration appeared to have little or no effect on NLP\textsubscript{Pya} and NLP\textsubscript{Sscl} binding.

A Limit of Detection (LoD) was calculated for each NLP assay using the method described by Armbruster and Pry (2008). The LoD for NLP\textsubscript{Pya} using rabbit anti-NLP\textsubscript{Pya} serum was 22 nM. Using the same anti-NLP\textsubscript{Pya} serum, the LoD for NLP\textsubscript{Bha} was 39 nM; for NLP\textsubscript{Sscl} the LoD was 49 nM; and for NLP\textsubscript{Fo} the LoD was 204 nM. A mean Limit of Binding (LoB) of OD\textsubscript{450} = 0.100 (equivalent to 8 nM NLP) was also calculated for all four NLP assays, based on background binding of anti-NLP\textsubscript{Pya} serum to wells coated with PBS only (Armbruster and Pry, 2008).
Figure 3.5. Summary of hyperimmune anti-NLP<sub>Py</sub> rabbit serum binding to various NLP coat concentrations. Serial dilutions of NLP ranging from 8.0 µM to 0.125 µM were coated on a 96-well plate. Serial dilutions of anti-NLP<sub>Py</sub> rabbit serum ranging from 1/300 to 1/153600, as well as pre-immune rabbit serum (Pre; undiluted) and PBS, were applied as primary antibody and detected using goat-anti-rabbit HRP in a dilution of 1/5000. Response of serum to select dilutions (8 µM, 1.0 µM and 0.125 µM) of each NLP are shown.
3.3.6. IC50s of anti-NLP<sub>pya</sub> serum with respect to NLPs

The affinity of anti-NLP<sub>pya</sub> serum binding to the NLPs was evaluated by CI-ELISA and calculated as the half-maximal inhibitory concentration (IC50) for each NLP (see Figure 3.6). Using data from checkerboard ELISAs (see Figure 3.5), working concentrations for anti-NLP<sub>pya</sub> serum were determined with respect to each NLP being evaluated. IC50s were calculated using a log(inhibitor) vs. response model

\[
Y = \text{Bottom} + \frac{[\text{Top}\,\text{-Bottom}]}{[1+10^{\text{X\,\text{-LogIC50}}}]} 
\]

in the software program GraphPad Prism for Mac 5.00. CI-ELISAs were performed a minimum of three times for the NLPs; as such, a mean IC50 for each NLP was calculated from IC50s generated from individual CI-ELISAs (presented in Figure 3.6). The IC50 for NLP<sub>pya</sub> using rabbit anti-NLP<sub>pya</sub> serum was 121 nM. Using the same anti-NLP<sub>pya</sub> serum, the IC50 for NLP<sub>bha</sub> was 10.5 nM; for NLP<sub>scl</sub> the IC50 was 11.3 nM; and for NLP<sub>fo</sub> the IC50 was 669 nM.
Figure 3.6. IC50s of NLPs in relation to anti-NLP<sub>Pya</sub> rabbit serum binding.

Microtitre plates were coated with varying concentrations of NLP as listed below. Varying concentrations of rabbit serum (listed below) were pre-incubated with serial dilutions of the coated NLP before being applied to blocked plates. Plates were probed with goat anti-rabbit-HRP as secondary antibody and developed for 10 minutes using TMB substrate. Background was determined by applying PBS to coated, blocked wells in place of rabbit serum. Coat and anti-NLP<sub>Pya</sub> rabbit serum concentrations (respectively) were: A, NLP<sub>Bha</sub> 0.125 µM and 1/20 000; B, NLP<sub>Fo</sub> 1.0 µM and 1/1200; C, NLP<sub>Pya</sub> 0.125 µM and 1/20 000; D, NLP<sub>SscI</sub> 0.5 µM and 1/20 000. OD<sub>450</sub> listed above represent means of background-subtracted triplicates ± SEM.
3.4. Discussion

The genes for NLP\textsubscript{Bha}, NLP\textsubscript{Fo} and NLP\textsubscript{Ssc} were cloned for expression into the vector pET28b+ and expression strain \textit{E. coli} BL21 (DE3). It was determined previously that NLP\textsubscript{Pya} showed excellent expression in this system, in the order of 10-20 mg/L cell culture (Fjällman, 2008). The same levels of expression were seen for the newly cloned NLPs. Expression and purification of recombinant NLP\textsubscript{Pya} in native form was unsuccessful because the expressed proteins aggregated as inclusion bodies within the \textit{E. coli} expression system (Fjällman, 2008). Due to the aggregatory nature of NLPs, it was reasonable to assume that the same problem would accompany expression and purification of NLP\textsubscript{Bha}, NLP\textsubscript{Fo} and NLP\textsubscript{Ssc}. Sonication and purification in denaturing conditions (8 M urea) was a successful solution to this issue; any protein present in inclusion bodies was broken up by the sonic dismembranator, and stayed solubilized in the urea. 8 M urea does not interfere with the function of a Ni-NTA column, so IMAC purification was also successful.

Following purification, the NLPs were refolded gradually during desalting in PBS via dialysis. Some protein aggregation occurred during this refolding process, as evidenced by visible precipitate forming in the dialysis tubing. In fact, it was not possible to concentrate NLP past \textasciitilde 10 \textmu M in PBS without precipitation occurring. Precipitates were easily removed by centrifugation, but the remaining soluble protein was of very low concentration (\textlesssim 2 \textmu M). As dialysis in other buffers did not affect the NLPs solubility, it was determined that working concentrations of 10 \textmu M or less were required (results not shown). SEC profiles for NLP\textsubscript{Bha}, NLP\textsubscript{Fo} and NLP\textsubscript{Pya} all showed the proteins eluting from a size exclusion column as monomers. However, the majority of NLP\textsubscript{Ssc} eluted as a
dimer, consistent with a protein size of ~50 kDa (twice that of NLP\textsubscript{Scl}). A second minor fraction eluted as a 25 kDa protein; this was monomeric NLP\textsubscript{Scl}. Based on the A\textsubscript{280} of both fractions, it appears that greater than 85% of NLP\textsubscript{Scl} is present as a dimer, while less than 15% of it is monomeric. This is perhaps not surprising, given the propensity of NLPs to aggregate and NLP\textsubscript{Scl}’s very high instability index of 42.48, classifying it as ‘unstable’. The NLP\textsubscript{Scl} dimer appears to be somewhat stable in solution, in that it forms no larger aggregates after the initial dialysed precipitate has been removed and can be maintained at a working concentration of 8 \mu M.

Previously, rabbits were immunized with recombinant NLP\textsubscript{Py} and their sera were found to have very high anti-NLP\textsubscript{Py} titre (Fjällman, 2008). Given that the GHRHDWE (NLP-heptapeptide) region of NLP\textsubscript{Py} was found to be the most immunogenic part of the protein (Fjällman, 2008), it was hypothesized that the rabbit serum bound NLP\textsubscript{Py} primarily in this conserved region. This possibility was explored through FPIA analysis of anti-NLP\textsubscript{Py} serum binding to the NLP-heptapeptide alone. A marked signal increase was seen when the serum was mixed with NLP-heptapeptide conjugated to a fluorophore (5-FAM-NLP-heptapeptide); this signal decreased as the concentration of serum decreased, indicating that antibodies in the serum bound the 5-FAM-NLP-heptapeptide molecule. An attempt was made to quantify this binding ability through a CI-FPIA; serum was pre-incubated with free NLP-heptapeptide and then tracer was added. However, no inhibition was seen, indicating that the serum had a much higher affinity for the 5-FAM-NLP-heptapeptide conjugate than the free NLP-heptapeptide. Given that the heptapeptide is only seven amino acids long, it is plausible that conjugation to 5-FAM changed the conformation and/or lipophilicity of the heptapeptide thus increasing its
affinity; alternatively, conjugation may have improved its accessibility to the serum paratopes while linked to a larger molecule.

Since the anti-NLP<sub>pya</sub> serum appeared to bind the NLP-heptapeptide, it would therefore follow that anti-NLP<sub>pya</sub> rabbit serum would have the ability to bind other NLPs. This theory was tested and confirmed using immunoblotting. Anti-NLP<sub>pya</sub> serum was shown to bind NLP<sub>Bha</sub> and NLP<sub>sscl</sub> in immunoblot format. The bands produced by the serum binding to NLP<sub>Bha</sub> and NLP<sub>sscl</sub> were much lighter than the band produced by the serum binding to NLP<sub>pya</sub>. This could be due to the polyclonal nature of the rabbit serum; while the serum antibodies would be able to bind NLP<sub>pya</sub> on all surface-exposed immunogenic structures, only serum antibodies binding the conserved structures on the NLP<sub>pya</sub> surface (such as NLP-heptapeptide) would have the ability to bind other NLPs. Thus, the serum would appear to bind NLP<sub>pya</sub> more strongly as a result of an avidity effect arising from epitopes unique to NLP<sub>pya</sub>. No binding to NLP<sub>Fo</sub> could be shown by immunoblot, indicating that the serum did not bind NLP<sub>Fo</sub> at all, or did not bind it strongly enough for confirmation in this experimental format. As the NLP-heptapeptide is most definitely present in NLP<sub>Fo</sub> (Nelson et al., 1998), a lack of binding would likely be due to the structure not being exposed, either fully or in part, on the surface of the protein; this could be confirmed with x-ray crystallographic data of the NLP<sub>Fo</sub> protein structure.

The ability of rabbit anti-NLP<sub>pya</sub> serum to bind various NLPs was compared by checkerboard and competition-inhibition ELISAs. Using the more sensitive ELISA format it was shown that the anti-NLP<sub>pya</sub> serum did bind NLP<sub>Fo</sub>, albeit at a reduced capacity compared to the other NLPs. NLP<sub>Fo</sub> had the greatest calculated LoD value of all
NLPs tested (204 nM) and required the greatest concentration of serum (a 1/1200 dilution) for CI-ELISAs. NLP<sub>Fo</sub> also had the highest IC50 of all four NLPs tested; at 669 nM, it was six times greater than the next-highest IC50 (NLP<sub>Pya</sub>) and 64 times higher than the lowest IC50 (NLP<sub>Bha</sub>).

The anti-NLP<sub>Pya</sub> serum showed similar binding attributes for NLP<sub>Bha</sub> and NLP<sub>Ssc</sub>. Both proteins required the same serum concentration for CI-ELISAs, a 1/20 000 dilution. Their LoDs were similar, with NLP<sub>Bha</sub> and NLP<sub>Ssc</sub> having LoD values of 39 nM and 49 nM respectively; thus the LoD of NLP<sub>Bha</sub> is five times lower than that of NLP<sub>Fo</sub>, and the LoD of NLP<sub>Ssc</sub> is four times lower. NLP<sub>Bha</sub> had an IC50 of 10.5 nM and NLP<sub>Ssc</sub> had an IC50 of 11.3 nM, indicating that anti-NLP<sub>Pya</sub> serum had very high affinity to both. This was surprising, given that the serum was not raised against either protein. Even more surprising is the juxtaposition of NLP<sub>Bha</sub> and NLP<sub>Ssc</sub> IC50s with that of NLP<sub>Fo</sub>, which had an IC50 60 times higher than either.

As one might expect, NLP<sub>Pya</sub> had the lowest LoD value of all NLPs tested. At 22 nM, it was an entire order of magnitude lower than the LoD for NLP<sub>Fo</sub>, and was approximately half the LoD for NLP<sub>Bha</sub> or NLP<sub>Ssc</sub>. The IC50 for NLP<sub>Pya</sub>, however, was actually higher than those for NLP<sub>Bha</sub> or NLP<sub>Ssc</sub>. At 121 nM, the IC50 for NLP<sub>Pya</sub> an order of magnitude higher the IC50s for NLP<sub>Bha</sub> or NLP<sub>Ssc</sub>. Given that the rabbit serum was raised against NLP<sub>Pya</sub>, this was quite unexpected. The discrepancy between the immunoblot and ELISA data could be due to the fact that the NLPs were in the denatured form, rather than their native form as was the case with the ELISA. Crystal structure and modeling comparisons have indicated that NLPs share a conserved tertiary structure in addition to conserved motifs like the NLP-heptapeptide (Ottmann et al., 2009). The
serum appeared to have a reduced ability to bind linearized NLP-heptapeptide (as seen in the FPIA experiments with free NLP-heptapeptide), which would reduce its ability to bind denatured NLP\textsubscript{Bha}, NLP\textsubscript{Pya} and NLP\textsubscript{Ssc} on the immunoblot at that site. Any conserved epitopes arising from tertiary structure would have been similarly affected. The polyclonal serum may have retained the ability to bind NLP\textsubscript{Pya} through epitopes unique to this protein, and thus would appear to have a higher affinity than to NLP\textsubscript{Bha} or NLP\textsubscript{Ssc} on the blot. The previously discussed avidity effect would present for the other NLPs too, but only in their native tertiary form and thus would only be observed in ELISA format.

The differences in binding and detection between the NLPs could be due to the position and surface exposure (partial vs. total) of the NLP-heptapeptide within the individual NLP structures. It is known that the NLP-heptapeptide motif in NLP\textsubscript{Pya} forms part of a negatively charged cavity on the protein surface which helps to coordinate a divalent cation, likely Ca\textsuperscript{2+} (Ottmann et al., 2009). This cavity may not be large enough to fully accommodate the paratope structure of a serum IgG, which may account for the anti-NLP\textsubscript{Pya} serum lower affinity to NLP\textsubscript{Pya} itself (compared to NLP\textsubscript{Bha} and NLP\textsubscript{Ssc}). Without detailed knowledge of the tertiary structures of NLP\textsubscript{Bha} and NLP\textsubscript{Ssc}, it is difficult to speculate what specific surface structures account for the differences in binding strength. The dimerization of NLP\textsubscript{Ssc} provides another confounding factor, in that the structure and orientation of the dimer is also currently unknown. It is, therefore, unknown how the NLP-heptapeptide motifs are positioned within the dimer and if their positions relative to each other could help binding through an avidity effect, or hinder it through blockage of one or both epitopes.
Two main conclusions can be drawn from these experiments. The first is that rabbit serum raised against one protein target, NLP\textsubscript{Pya}, was found to have specific and quantifiable cross-reactivity to other related proteins. The second is that this cross-reactivity appears to be due, at least in part, to the conserved heptapeptide motif present in all proteins bound. Due to the finite nature of anti-serum, the scope of the rabbit anti-NLP\textsubscript{Pya} serum as a major diagnostic and preventative tool was limited. Thus, the NLP-heptapeptide made a plausible antigenic target from which to raise cross-reactive, recombinant monoclonal binders for the detection and neutralization of multiple NLPs. This possibility was explored in Chapter 4.
4. **SELECTION AND ENGINEERING OF MONOCLONAL ANTIBODIES FOR DETECTION AND NEUTRALIZATION OF NEP1-LIKE PROTEINS**

4.1. Introduction

In the humoral immune response, B cell development is intrinsic to the production of fully mature, high affinity antibodies by the immune system. It is during the maturation from pro-B cell to pre-B cell to immature B cell that the variable regions of antibodies are synthesized. $V_H$, $D_H$ and $J_H$ genes rearrange and join with a $C_\mu$ gene to form the HC, then $V_L$ and $J_L$ genes rearrange and join with a $C_\kappa$ or $C_\lambda$ gene to form the LC. Antibody diversity is generated during this process through the combinatorial joining of multiple germ-line segments, as well as through junctional flexibility and addition of $P$- and $N$-nucleotides. After gene rearrangement, the process of hypersomatic mutation acts as an *in vivo* affinity maturation mechanism. By the time class-switching from $\mu$ to immunoglobulin isotypes $\gamma$, $\alpha$, $\delta$ and $\epsilon$ occurs, a robust and highly sensitive antibody response has developed against the immunogen (Goldsby et al., 2003).

The results from Chapter 3 showed that serum from rabbits immunized against one NLP was capable of binding three others through a conserved heptapeptide motif known as the NLP-heptapeptide. In this chapter, mice were immunized with the NLP-heptapeptide for the express purpose of raising a monoclonal antibody capable of binding multiple NLPs in a quantifiable fashion. This mAb was evaluated in several different formats to explore the advantages and disadvantages of its production as various antibody species. The end result was a specific and cross-reactive IgG capable of binding multiple NLPs with similar levels of affinity.
4.2. Methods and Materials

4.2.1. NLP-heptapeptide synthesis and immunization in mice

NLP-heptapeptide (GHRHDWE) was synthesized with a small linker of five amino acids (SGSGC) resulting in 12 amino acid peptide, GHRHDWESGSGC. The peptide was manufactured in its free form, and conjugated through the linker to keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) and 5-carboxy fluorescein (5-FAM) (Immunoprecise Antibodies, Victoria, BC).

Four 53-day-old BALB/c mice (Charles River Laboratories, Senneville, QC) were immunized intraperitoneally (IP) with GHRHDWE-KLH conjugate in Complete Freund’s adjuvant (Sigma-Aldrich, Oakville, ON) at a dose of 25 µg. Mice were given intravenous (IV) boosts of 25 µg GHRHDWE-KLH conjugate in Incomplete Freund’s adjuvant (Sigma-Aldrich) for a total of two boosts. All immunizations occurred in volumes of 125 µL. Mice were test bled ten days after the second boost (third overall immunization; 51 days after first immunization) and titres to GHRHDWE-BSA were evaluated. Based on their low cross-reactivity to GHRHDWE-BSA, mice 1 and 4 were chosen for a final IV boost. Ten µg GHRHDWE-KLH in 100 µL sterile saline was administered via the lateral tail veins. Both mice died from anaphylactic shock post-injection; as such, spleens and lymphocytes were harvested immediately after the final boost. Remaining mice 2 and 3 received two more IP boosts, following the same dose and schedule as before. Mice were test bled again after the fourth boost (fifth overall immunization; 93 days after first immunization) and titres to GHRHDWE-BSA were re-evaluated. The mice were euthanized and their sera, spleens and lymphocytes were
harvested. All boosts, including final IV boost, were administered in intervals of three weeks (Immunoprecise Antibodies).

4.2.2. Fusion, screening and isotyping of hybridoma clones

After harvesting, splenocytes from mice 1 and 4 were stimulated with 100 µg GHRHDWE-KLH in sterile culture in the presence of cytokines for four days; 5 mL per well were plated over 5 wells of a 6-well plate for a total culture volume of 25 mL. Prior to fusion, stimulated lymphocytes were Ficoll-Paque purified using Lymphoprep (STEMCELL Technologies Inc, Vancouver, BC). Lymphocytes were fused to SP2/0 myeloma cells using the PEG 1500 protocol by Roche (Laval, QC). Fusion was performed 71 days after the first immunization was administered. Hybridoma cells were grown in DMEM Complete media (dipeptide glutamine, sodium pyruvate) plus HT supplement (Mediatech, Manassas, VA), Penicillin/Streptomycin HyQ (Thermo Fisher Scientific, Ottawa, ON), and 10% fetal bovine serum (FBS; Thermo Fisher Scientific) (Immunoprecise Antibodies).

948 hybridoma clones were screened for specificity by indirect ELISA. Plates were coated with 1.0 µg per well of GHRHDWE-BSA or 0.5 µg/well human transferrin (HT) and dried down overnight at 37°C. Plates were blocked with 100 µL per well 3% skim milk in PBS. Tissue culture (TC) supernatant from hybridoma clones was applied as primary antibody, 100 µL neat per well. Goat anti-mouse IgG + IgM (H+L)-HRP (100µL; Jackson ImmunoResearch, West Pike, PA) diluted 1/10 000 in PBST was applied to each well as secondary antibody. Plates were developed with 50 µL TMB substrate (BioFx, Owings Mills, MD) and quenched after ten minutes using 50 µL 1.0 M
HCl before reading at 450 nm. All steps were incubated for one hour at room temperature unless otherwise stated (Immunoprecise Antibodies).

The isotypes of antibodies secreted by 74 hybridoma clones were determined by trapping ELISA. Goat anti-mouse IgG/IgM trapping antibody (Jackson ImmunoResearch) was diluted 1/10 000 in carbonate buffer and a 96-well microtitre plate was coated 100 µL/well overnight at 4°C. After aspirating, plates were washed once with PBST. TC supernatant (100µL) was applied as primary antibody, one clone per microtitre well, as well as several unrelated TC supernatants (SP2/0, X63, mouse 2B4.4 mAb, mouse anti-BSA mAb, mouse anti-HT mAb) and mouse serum diluted 1/500 (preimmune and mouse anti-GHRHDWE-KLH from test bleed). Primary antibodies were incubated for one hour at room temperature, then plates were aspirated and washed five times over 30 minutes with PBST. Secondary antibodies goat anti-mouse IgG (mixture of monoclonal anti-IgG1, 2a, 2b, 3) Fc-HRP (Jackson ImmunoResearch) and goat anti-mouse IgMµ-HRP (Pierce) were diluted 1/25 000, applied to appropriate wells at 100 µL per well, and incubated at room temperature for one hour. Plates were aspirated and washed five times over 30 minutes with PBST, then developed using 50 µL TMB substrate (BioFx). Plates were quenched with 50 µL 1.0 M HCl and read at 450 nm; a positive result was determined by a signal of two or three times background. This assay was repeated once. Concentrations of secreted antibody were estimated to be 100 µg/mL TC supernatant (Immunoprecise Antibodies).
4.2.3. Anti-NLP indirect ELISA

Plates were coated with 100 µL per well of 1 µM NLP for one hour at 37°C, then washed and blocked overnight at 4°C with 200 µL per well 1.5% casein in PBS. Primary antibodies and appropriate controls were applied 100 µL per well and plates were incubated for one hour at room temperature. Appropriate secondary antibodies were applied 100 µL per well and incubated for one hour at room temperature. Plates were developed using 100 µL two-component TMB substrate (KPL, Guelph, ON) before quenching with 100 µL 0.1 M H₂SO₄ and reading at 450 nm. Plates were washed three times with PBST 300 µL per well between each step. Various anti-NLP antibodies were tested as primary antibodies with the following specifications below.

4.2.3.1. Using IgM as primary antibody

TC supernatants from 70 hybridoma clones were diluted 1/10 in PBS and applied in duplicate as primary antibody, while DMEM + 10% FBS diluted 1/10 in PBS, rabbit anti-NLPₚₚₚ serum diluted 1/10 000, mouse anti-GHRHDWE serum diluted 1/5000 in PBS and PBS were applied 100 µL per well in triplicate. Goat anti-mouse IgG + IgM (H+L)-HRP (monoclonal mixture; Jackson ImmunoResearch) diluted 1/5000 and goat anti-rabbit-HRP (Pierce) diluted 1/6000 were applied as secondary antibody. Plates were developed for five minutes before quenching.

4.2.3.2. Using F(ab’)μ as primary antibody

F(ab’)μ samples were diluted 1/10 in PBS and applied in duplicate as primary antibody, as well as DMEM + 10% FBS diluted 1/10 in acetate buffer pH 4.5, DMEM + 10% FBS diluted 1/10 in PBS and PBS in sextuplicate. Goat anti-mouse IgG + IgM
(H+L)-HRP (Jackson ImmunoResearch) diluted 1/5000 in PBS was applied as secondary antibody. Plates were developed for fifteen minutes before quenching.

4.2.3.3. Using scFv as primary antibody

_E. coli_ HB2151 (6B1) enzymatic cell lysate, _E. coli_ HB2151 (untransformed) cell lysate and PBS were applied neat as primary antibody. Mouse anti-c-myc-HRP (Abcam) diluted 1/10 000 was applied as secondary antibody. Plates were developed for 20 minutes before quenching.

4.2.3.4. Using IgG as primary antibody

_N. benthamiana_ crude extract from leaves infiltrated with _A. tumefaciens_ (6B1 HC and LC and p19) bacterial cocktail or with _A. tumefaciens_ (p19) were applied neat as primary antibody, as well as PBS and rabbit anti-NLP_Pya serum diluted 1/10 000. Goat anti-human (H+L)-HRP (Pierce) diluted 1/10 000 and goat anti-rabbit-HRP (Pierce) diluted 1/6000 were added as secondary antibody. Plates were developed for 20 minutes before quenching.

4.2.4. IgM fragmentation to F(ab’)µ antibodies

24 IgMs were each purified from 2.0 mL TC supernatant using Protein L spin columns according to manufacturer’s instructions (Pierce) and dialysed overnight at 4°C in acetate buffer pH 4.5. Pepsin was added to each dialysed fraction to a final concentration of 5 µg/mL, estimating an antibody:enzyme ratio of 20:1 (based on IPA estimates of 100 µg IgM/mL TC supernatant). Reaction mixtures were incubated in a waterbath at 37°C for 12 hours, then the reaction was stopped by the addition of 2.0 M Tris to a final pH of 8.5. Cysteine was added to the reaction mixtures to a final
concentration of 10 µM and incubated in a waterbath at 37°C for two hours. Fractions were dialysed overnight at 4°C against PBS using dialysis tubing with a 50 kDa molecular cut-off, then stored at 4°C.

4.2.5. scFv cloning from hybridoma RNA

RNAlater (Qiagen) preparations of hybridoma cells were centrifuged at 5000 x g for 10 minutes, then homogenized in 600 µL buffer using a Qiashredder (Qiagen). Total RNA was isolated with an RNeasy Minikit (Qiagen) according to the manufacturer’s instructions, and RNA was eluted using 50 µL diethylpyrocarbonate (DEPC)-treated water. Complementary DNA (cDNA) was synthesized using a First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer’s instructions; 5 µL purified RNA was primed with oligo(dT) provided by the kit. \(V_H\) and \(V_L\) genes were amplified from cDNA using the following reaction mixtures: 2.5 U Taq DNA polymerase (New England Biolabs), 5 µL supplied buffer with KCl, 1.5 mM MgCl2, 10 nM dNTPs, 2 µM forward primer mix, 2 µM reverse primer mix and 2 µL cDNA template in a reaction volume of 50 µL. The thermal cycle used to amplify the genes was: 3 min at 92°C; five cycles of 30 s at 92°C, 60 s at 55°C, 60 s at 72°C; 30 cycles of 30 s at 92°C, 60 s at 63°C, 60 s at 72°C; five min at 72°C. Primer sequences were those listed in Burmester and Plückthun (2001), modified to introduce XhoI restriction sites on \(V_H\) forward primers and \(V_L\) reverse primers for insertion in expression vector pIT2. PCR products were quantified on a 1% agarose gel using a Low DNA Mass Ladder (Invitrogen) then cloned into the pCR2.1-TOPO vector using a using a TOPO TA Cloning Kit (Invitrogen) according to the manufacturer’s instructions. Reaction mixture (1 µL) was transformed into 25 µL \(E.\)
coli Top10F cells by electroporation with the following parameters: 1 mm cuvette, 1800 V, 25 µF and 200 Ω. Cells were plated on LB agar plus 50 µg/mL carbenicillin and 40 µL per plate of 40 mg/mL Xgal absorbed on the plate’s surface, and incubated overnight at 37°C. Using blue/white screening, positive (white) transformants were selected for colony PCR using V\textsubscript{H} and V\textsubscript{L} specific primers. The reaction mixtures used were: 1.25 U Pfu DNA polymerase (Fermentas), 2.5 µL supplied buffer, 10 nM dNTPs, 4 µM forward primer mix, 4 µM reverse primer mix and 1 µL source DNA in a reaction volume of 25 µL. The thermal cycle used to amplify the genes was: 10 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C; five min at 72°C. A minimum of five positive transformants for each V\textsubscript{H} and V\textsubscript{L} gene from each clone was sent for sequencing. Sequences were analysed using the ClustalX alignment tool (Larkin et al., 2007) and sequences showing consensus were selected for subcloning into expression vector pIT2. V\textsubscript{H} sequences and pIT2 vector were double digested with NcoI and XhoI restriction enzymes, run on a 1% agarose gel, then extracted and purified using a QIAquick gel extraction kit (Qiagen) to isolate V\textsubscript{H} gene fragments and fully digested pIT2 vector. Vector and gene samples were quantified on an agarose gel using a Gel Doc system and software (Bio-Rad). Ligation mixtures of 10 µL were set up using a 3:1 stoichiometric ratio of gene to vector, plus 1 U T4 ligase in supplied buffer (Fermentas); mixtures were ligated overnight at 16°C, transformed into E. coli Top10 cells via electroporation and plated on LB agar plus carbenicillin (75 µg/mL). Colony PCR was used to screen for positive transformants, which were cultured overnight at 37°C in 10 mL LB plus carbenicillin cultures (75 µg/mL), and from which plasmids were harvested using a Miniprep kit (Qiagen). This procedure was repeated to insert V\textsubscript{L} sequences into pIT2.
containing V_{H} sequences, with the following exception: V_{L} sequences were double
digested with XhoI and NotI restriction enzymes and pIT2 vector plus V_{H} sequences was
double digested with SalI and NotI. pIT2 vector containing both V_{H} and V_{L} sequences
was transformed into E. coli HB2151 by electroporation. Positive transformants were
screened by colony PCR using M13 primers, then verified by sequencing.

4.2.6. scFv expression in E. coli HB2151

Starter cultures of 5 mL 2xYT (16 g peptone, 10 g yeast extract, 5 g NaCl per L)
containing 50 µg/mL carbenicillin and 1% glucose were grown overnight at 37°C and
200 rpm. Starter cultures were added to 1 L of 2xYT plus 50 µg/mL carbenicillin and 1%
glucose and grown at 37°C and 200 rpm until an OD_{600} of 0.7 was obtained. Cells were
centrifuged at 2000 x g for 10 min at room temperature and supernatant was discarded.
Cell pellets were resuspended into 1L 2xYT plus 50 µg/mL carbenicillin, 500 µM IPTG
and 0.1% glucose and incubated at room temperature with shaking (200 rpm) for 24 h.
Cells were harvested by centrifugation at 8000 x g for 10 min and stored at -20°C.

4.2.7. scFv extraction and purification

4.2.7.1. Lysozyme extraction of native scFv

Cell pellets were resuspended in 100 mL ice-cold lysis buffer (50 mM Tris-HCl
pH 8.0, 25 mM NaCl, 2 mM EDTA); 1 mL of 100 mM phenylmethylsulfonyl fluoride
(PMSF) and 200 µL of 1 M dithiothreitol (DTT) were added to the ice-cold solution.
Freshly prepared lysozyme (5 mL; 3 mg/mL in nanopure water) was added and the
mixture was incubated at room temperature for 40 min with occasional shaking. DNase I
(250 µL; 15 U/µL in 1 M MgCl₂) was added and mixture was incubated at room temperature for 30 min, then centrifuged at 5000 x g at 4°C for 20 min twice. Supernatant was dialysed overnight at 4°C against Protein L binding buffer (20 nM sodium phosphate pH 7.2 plus 150 nM NaCl) using dialysis tubing with a 12-14 kDa molecular weight cut-off. Supernatant was loaded on a 5 mL Hi-Trap Protein L column and purified using an ÄKTA FPLC system (GE Healthcare Bio-sciences). After washing, scFv was eluted with 50 mL of 0.1 M sodium citrate pH 3.0 and neutralised with 100 µl/mL eluent 1 M Tris-HCl pH 8.0. Eluted fractions were analysed by SDS-PAGE and immunoblot.

4.2.7.2. Sonication of denatured scFv

Frozen cell pellets were solubilized in 20 mL denaturing IMAC buffer (10 mM HEPES plus 500 mM NaCl and 8 M urea, pH 7) and sonicated using a Model 550 Sonic Dismembranator (Fisher Scientific). Cell lysate was centrifuged to separate soluble and insoluble cell fractions. Soluble cell fraction was loaded on a 5 mL Hi-Trap Ni-NTA column and purified using an ÄKTA FPLC system (GE Healthcare Bio-sciences). Elution was performed using a stepwise gradient of imidazole in denaturing IMAC buffer (10 mM HEPES plus 500 mM NaCl, 8 M urea and 500 mM imidazole, pH 7); imidazole concentrations used were 50 mM, 75 mM, 100 mM, 150 mM, 200 mM, 250 mM and 500 mM. Eluted fractions were analysed by SDS-PAGE and immunoblot.

4.2.8. Anti-scFv immunoblotting

_E. coli_ HB2151 cell lysates and FPLC fractions containing scFv underwent electrophoresis on a reducing 15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and were transferred to PVDF membrane (Bio-Rad). For immunoblot analysis,
membranes were blocked in 3% skim milk (EMD Chemical) overnight at room
temperature. Blots were probed with mouse anti-c-myc antibody (Abcam) diluted 1/5000
in PBS, then probed with goat anti-mouse-AP secondary antibody (Pierce) diluted 1/4000
in PBS and developed for 30 min. Alternatively, blots were probed with mouse anti-
6xHis-AP antibody (Abcam) diluted 1/5000 in PBS and developed for 20 min. All
antibodies were incubated for one hour at room temperature plus shaking. Blots were
washed three times for five minutes each with PBST between each step, and developed
with 10 mL NBT-BCIP substrate (Pierce).

4.2.9. Cloning scFv 6B1 to IgG format

Based on its coding sequences, scFv 6B1 was redesigned as full a length IgG. 6B1
V<sub>H</sub> and V<sub>L</sub> coding sequences were inserted into separate p105 expression cassettes
(Garabagi et al., 2012) containing human G1 and κ constant regions for expression in <i>N.
benthamiana</i>. Restriction sites were added to the 5’ and 3’ ends of both variable region
coding sequences for future subcloning: AsiSI and BstEII for 6B1 V<sub>H</sub> and AsiSI and
XhoI sites for 6B1 V<sub>L</sub>. An <i>Arabidopsis</i> basic chitinase signal sequence (ABC) was also
incorporated directly upstream from the V<sub>H</sub> and V<sub>L</sub> to target coexpressed heavy and light
chains to the endoplasmic reticulum of <i>N. benthamiana</i> cells (see Figure 4.1). Signal
sequence cleavage from translated peptides was predicted using the PrediSi tool online
(Hiller, 2003). V<sub>H</sub> and V<sub>L</sub> coding sequences were codon optimized for expression <i>in
planta</i> using the backtranslation tool online (Fischer, 2013). Splice sites were predicted
using the NetPlantGene tool online (Hebsgaard et al., 1996) and corrected by codon
substitution. Finally, the miRBase online was used to check coding sequences for
microRNAs (Kozomara and Griffiths-Jones, 2011). Genes were synthesized and inserted into p105 by Genewiz Inc (South Plainfield, NJ). Vectors were transformed individually into *Agrobacterium tumefaciens* strain 542 by electroporation: 100 ng DNA was added to 20 µL competent cells, transferred to a 1 mm cuvette and pulsed at 25 µF, 200 Ω and 2400 V. Cells were recovered in 500 µL LB for 1.5 hours at 28°C then plated on LB agar plus 50 µg/mL each of carbenicillin and rifampicin and incubated for three days at 28°C.

**Figure 4.1. Expression cassette design for 6B1 reformatting to IgG in p105.**

Expression cassettes were situated on the T-DNA region of binary vectors. *Arabidopsis* basic chitinase signal sequence (ABC) preceded both variable region coding sequences. Red text indicates an inserted restriction site. p105 diagram modified from Garabagi et al., 2012a.

4.2.10. IgG expression and purification in *N. benthamiana*

Cultures (10 mL) of *A. tumefaciens* 542 containing V\(_H\), V\(_L\) and p19 coding sequences were each inoculated in LB plus 50 µg/mL each of carbenicillin and rifampicin
and incubated for 16 h at 28°C with shaking (220 rpm) until each culture reached an 
OD$_{600}$ of 0.8-2.0. Based on their OD$_{600}$, the volume of culture required for a final OD of 
0.2 for each *A. tumefaciens* culture was calculated. Cultures were combined in the 
calculated volumes and centrifuged at 2000 x g for 10 minutes at room temperature, then 
resuspended in 10 mM 1-(N-morpholino)ethanesulfonic acid (MES) pH 5.5 containing 10 
mM MgSO$_4$ for coexpression. p19 culture was also resuspended in infiltration buffer by 
itsel, also at a final OD$_{600}$ of 0.2. Using a 1 mL needleless syringe (Restek, Guelph, ON), 
*N. benthamiana* leaf tissue was infiltrated with 500 µL per spot of IgG + p19 mixture or 
p19 alone (as a negative control). Plants were grown for seven more days, then infiltrated 
leaves were harvested and stored at -20°C.

Leaf tissue was mechanically homogenized in Protein G binding buffer (0.2 M 
sodium phosphate, pH 7.0) using a Polytron blender, then filtered and centrifuged to 
remove insoluble cell matter. The soluble cell fraction was loaded on a 5mL Hi-Trap 
Protein G column (GE Healthcare Bio-sciences). After washing, IgG was eluted with 50 
ml of 0.1 M glycine pH 3.0 and neutralised with 100 µl/mL of 1 M Tris-HCl pH 9.0. 
Eluted fractions were analysed by reducing and nonreducing SDS-PAGE and 
immunoblotting. Immunoblots were performed as described in 4.2.8 with the exception 
that they were probed with goat anti-human (Fab)-AP (Pierce) diluted 1/1000 in PBS. 
Fractions containing IgG were dialysed against PBS using 50 kDa MWCO tubing and 
quantified by spectrophotometric readings at 280 nm on a Nanodrop 2000c (Thermo 
Scientific).
4.2.11. Anti-NLP immunoblotting

SDS-PAGE and immunoblot were run as described in 3.3.4 with the following modifications: 2 µg protein were loaded per well; His-tagged \( V_\beta H \) was not among the samples analysed; the immunoblots were probed with 40 nM 6B1 IgG primary antibody and goat anti-human (Fab)-AP secondary antibody diluted 1/1000 in PBS.

4.2.12. Surface plasmon resonance (Biacore)

Using a Biacore T200 machine and a CM5 S sensor chip, a variety of different experimental conditions were tested to attempt to show 6B1 IgG binding to NLPs. Various buffers, surface densities, coating ligand, and antigen concentration were tested (see Table 4.1). 6B1 was immobilized with 10nM sodium acetate pH 4.5 and an amine coupling kit (GE Healthcare). A mouse anti-human \( \beta \) microglobulin IgG was coated on a second flow cell to measure background and the chip was blocked with 1 M ethanolamine pH 8.5. Antigen (40 µL) was injected at a flow rate of 20 µL/minute. NLPs were immobilized on a CM5 chip using a Biacore 3000 machine. Coupling was performed at pH 4.5 and ethanolamine was injected on a separate flow cell as a background reference. Using a Fab Preparation Kit (Pierce), Fab was prepared from 6B1 IgG and 100 µL was injected over the NLP-coated chip. A Human Antibody Capture Kit (GE Healthcare Bio-sciences) was coated on the chip at pH 5.0 to a surface density of 11 000 RU and 6B1 IgG was bound by the capture antibodies at a surface density of 5400 RU prior to test injections with NLP. All NLPs were purified using as described in 3.2.2.
Table 4.1. Summary of Biacore experiments. Using a Biacore T200 machine (GE Healthcare), various experimental conditions were combined to attempt to measure the capacity of 6B1 IgG to bind NLPs. Ligands were immobilized to the chip surface by amine coupling and surface density was measured in response units (RU). The maximum analyte binding capacity of the chip surface ($R_{\text{max}}$) was either calculated based on ligand and antigen sizes and surface density (theoretical, $R_{\text{max}}(T)$) or measured by the machine (experimental, $R_{\text{max}}(E)$). For experiments 13 to 16, an antibody capture kit was employed to facilitate correct IgG orientation and binding. A mouse anti-human IgG was immobilized on the chip surface by amine coupling (11 000 RU); 6B1 IgG was bound by the capture antibody and antigen was injected on the chip surface as listed. A fresh capture-6B1 surface was prepared for experiment 16 and test injections of NLP$_{\text{Bha}}$ were performed in concentrations of 0.25, 0.5, 1.0 and 2.5 µM. ^ denotes the same condition was repeated from the previous experiment.

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<td>^</td>
<td>^</td>
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<td>^</td>
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<td>NLP$<em>{\text{Fo}}$, NLP$</em>{\text{Pya}}$</td>
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4.2.13. Anti-NLP CI-ELISA

4.2.13.1. Using scFv antibodies

CI-ELISA was performed as described in 3.2.7 with the following changes: E. coli HB2151 enzymatic cell lysate was pre-incubated neat with serial dilutions of each NLP; mouse anti-c-myc-HRP (Abcam) diluted 1/10 000 in PBS was added to the wells as secondary antibody; plates were developed using two-component TMB substrate (KPL) and quenched using 0.1 M H$_2$SO$_4$ after 45 minutes.

4.2.13.2. Using IgG antibody

CI-ELISA was performed as described in 3.2.7 with the following changes: microtitre wells were coated with NLP$_{Bha}$ 0.25 µM, NLP$_{Fo}$ 1.0 µM, NLP$_{Pya}$ 1.0 µM or NLP$_{Ssc}$ 1.0 µM; 6B1 IgG diluted to 8 nM or 16 nM (for NLP$_{Ssc}$) in PBS was pre-incubated with serial dilutions of each NLP; goat anti-human (H+L)-HRP (Pierce) diluted 1/10 000 in PBS was added as secondary antibody; plates were developed using two-component TMB substrate (KPL) and quenched using 0.1 M H$_2$SO$_4$ after 30 minutes.

4.3. Results

4.3.1. Mouse serum titres in response to NLP-heptapeptide immunizations

Mice were test bled ten days after their second boost and serum responses to NLP-heptapeptide were measured by ELISA (see Figure 4.2). All four mice had a strong IgG response versus the coated GHRHDWE-BSA conjugate; sera OD$_{450}$ were ten times that of pre-immune serum tested, and 25 times that of PBS (plate background). Sera were also tested in a parallel ELISA using an unrelated peptide-BSA conjugate; serum OD$_{450}$ had negligible differences from those of pre-immune serum and PBS. Sera IgM binding
to GHRHDWE-BSA were also measured; serum IgM response was 25% to 35% of serum IgG response as measured by OD$_{450}$. After the second boost, mice 1 and 4 were given a final IV boost and sacrificed, while mice 2 and 3 were given two more boosts continuing the same schedule and dosage.

Remaining mice 2 and 3 were test bled a second time after their fourth boost and serum responses to NLP-heptapeptide were compared between the two test bleeds by ELISA (see Figure 4.3). IgG responses to NLP-heptapeptide increased marginally in the second test bleed, as did IgM responses. Thus, IgM responses of the mice remained in the order of 25% to 35% of the IgG response.

4.3.2. Hybridoma screening and isotyping

Seventy-one days after their first immunization, splenocytes from mice 1 and 4 were fused with myeloma cells to produce antibody-secreting hybridoma cells. 948 hybridoma clones were produced and subsequently screened to evaluate antibody binding, specificity, and isotype. All 948 hybridoma clones were screened on GHRHDWE-BSA and 164 top binders were identified. These 164 binders were re-screened on GHRHDWE-BSA, as well as human transferrin (HT; negative screening antigen). Positive clones (74) were identified, as well as 51 non-secreting or false positive clones and 39 clones recognizing HT which were considered to be non-specific binders. The 74 positive clones were isotyped via trapping ELISA; 4 clones stopped secreting and the remaining 70 clones were found to be IgM isotype.
Figure 4.2. Mouse IgG and IgM serum responses to NLP-heptapeptide 10 days after 2\textsuperscript{nd} boost. Test bleeds from all four mice were taken 10 days after their 2\textsuperscript{nd} boost with NLP-heptapeptide-KLH conjugate (51 days after first immunization). Microtitre plate wells were coated with 1 µg/well of NLP-heptapeptide-BSA conjugate or an unrelated peptide-BSA conjugate (UnRel). Serial dilutions of immune and preimmune sera ranging from 1/100 to 1/12800 were applied as primary antibody. Serum IgG and IgM responses were measured by using goat anti-mouse IgG Fc-HRP or goat anti-mouse IgM\textsubscript{\mu}-HRP secondary antibodies (respectively). Response of select serum dilutions (1/3200) shown.
Figure 4.3. Mouse IgG and IgM serum responses to NLP-heptapeptide after 2\textsuperscript{nd} and 4\textsuperscript{th} boosts. Test bleeds from mice 2 and 3 were taken after 4\textsuperscript{th} boost with NLP-heptapeptide-KLH conjugate (93 days after first immunization). Microtitre plate wells were coated with 1 µg/well of NLP-heptapeptide-BSA and serial dilutions of immune (from current and previous test bleeds) and preimmune sera (primary antibody) ranging from 1/100 to 1/12800. Serum IgG and IgM responses were measured by using goat anti-mouse IgG Fc-HRP or goat anti-mouse IgM\textsubscript{\mu}-HRP secondary antibodies (respectively). Response of select serum dilutions (1/3200) shown.
4.3.3. Hybridoma IgM binding and cross-reactivity to NLPs

To facilitate the clone selection process, IgM binding to NLPs was measured by indirect ELISA with diluted hybridoma TC supernatant. Figure 4.4 shows the relative binding strength of 70 hybridoma clones for each NLP. Binding to an NLP was considered significant if the observed OD$_{450}$ was three times the plate background or higher; plate background was assessed as the mean of triplicate treatments of DMEM + 10% FBS diluted 1/10 in PBS (containing no antibody). Forty-six clones were found to have significant binding to NLP$_{Bha}$, 32 to NLP$_{Fo}$, 40 to NLP$_{Pya}$ and 41 to NLP$_{Sscl}$. Based on their superior binding strength to all 4 NLPs, 24 clones were chosen to be maintained in cell culture for further testing, indicated in Figure 4.4 with the symbol *.

4.3.4. Relative hybridoma F(ab’)µ binding to NLPs

In order to approximate the monovalent binding capacity of an scFv, the 24 IgM clones were fragmented to F(ab’)µ antibodies. Fragmentation was successful, as determined on immunoblots by the presence of 40 kDa bands under reducing conditions and 65 kDa bands under nonreducing conditions, corresponding to the µ chain and F(ab’)µ species (respectively) for each antibody (results not shown). Figure 4.5 shows the relative binding strength of 24 F(ab’)µ preparations for each NLP. Binding to an NLP was considered significant if the observed OD$_{450}$ was two times plate background or higher; plate background was assessed as the mean of triplicate treatments of PBS applied as a primary treatment without antibody. Four clones were found to have significant binding to NLP$_{Bha}$, nine to NLP$_{Pya}$, none to NLP$_{Fo}$, and two to NLP$_{Sscl}$. Many clones showed binding that was higher than plate background but not high enough to be
Figure 4.4. IgM binding and cross-reactivity to NLPs. Continued next page.
Figure 4.4. IgM binding and cross-reactivity to NLPs. Microtitre plate wells were coated with 100 µL of 1 µM NLP. Tissue culture supernatants from 70 positive hybridoma clones and untreated DMEM + 10% FBS were diluted 1/10 in PBS and 100 µL of each dilution was applied as primary antibody. Goat anti-mouse IgG + IgM (H+L)-HRP was applied as secondary antibody. Wells containing untreated DMEM + FBS diluted 1/10 in PBS were used to calculate mean background absorbance. Values presented above are means of background-subtracted duplicates. * Indicate clones (24) selected for further study via fragmentation to F(ab’)µ; ^ Indicate clones (11) selected for scFv cloning.
Figure 4.5. F(\text{ab'})\mu binding and cross-reactivity to NLPs. IgMs were fragmented to F(\text{ab'})\mu by pepsin digestion and cysteine reduction followed by their ability to bind NLPs measured by indirect ELISA. Microtitre plate wells were coated with 100 μL of 1 μM NLP. F(\text{ab'})\mu preparations were diluted 1/10 in PBS and 100μL of each dilution was applied as primary antibody. Goat anti-mouse IgG + IgM (H+L)-HRP was applied as secondary antibody. Wells containing PBS only were used to calculate mean background absorbance. Values presented above are means of background-subtracted duplicates. * Indicates clone was selected for further study by scFv cloning.
considered significant; 22 clones had non-significant binding to NLP\textsubscript{Bha}, five for NLP\textsubscript{Fo}, all 24 for NLP\textsubscript{Pya} and 15 for NLP\textsubscript{SscI}. Eleven clones were selected for cloning to scFvs, indicated in Figure 4.4 with the symbol ^ and Figure 4.5 with the symbol *.

4.3.5. Amino acid sequences of V\textsubscript{H} and V\textsubscript{L} fragments for scFv clones

The V\textsubscript{H} and VL coding sequences for each antibody were determined by nucleotide sequence analysis. Due to the error-prone nature of PCR amplification, at least five clones per gene insert were analysed (minimum ten total per scFv clone). Sequences were compared at DNA and amino acid levels for homology to determine an apparent sequence; translated sequences were checked for canonical antibody structures, including cysteine pairs and recognizable complementarity-determining regions (CDRs). Translated consensus sequences that were recognized as antibodies are compared in Figure 4.6. All heavy chains showed distinct and diverse CDR sequences, particularly CDR3. Light chains showed very little diversity among CDRs and nearly reached consensus in CDR2 and CDR3. In fact, V\textsubscript{L} sequences for 7H4 and 8E2 differed by a single framework region amino acid (position 4, I vs. T), while V\textsubscript{L} sequences for 1D3 and 3H7 were identical.

Many translated light chain sequences did not correspond to recognizable antibody structures; these sequences are compared in Figure 4.7. All non-antibody V\textsubscript{L} sequences either showed consensus or differed from consensus by one or two amino acids. No cysteine pairs or recognizable CDRs were apparent in these translated sequences. The recognizable V\textsubscript{L} antibody sequence for scFv 6B1 was included at the bottom of the figure for contrast. Sequence similarity for all clones (6B1 included) is maintained for the first 22 amino acids after which 6B1 diverges from all sequences.
Figure 4.6. Jalview sequence alignment of conservation and consensus of amino acid sequences among 11 scFv clones. scFv sequence lengths (number of amino acids) are listed beside names. Conservation numbers (0-9, 10 = *) provide numerical scale for degree of physiochemical conservation at any given amino acid position, as calculated by Livingstone and Barton (1993). Quality provides a measure of the inverse likelihood of unfavourable mutations in the alignment. Consensus letters refer to amino acid homology present in more than two sequences for a position; A “+” indicates there are two or more conflicting homologies for that position. (Waterhouse et al., 2009.) A: scFv V_H translated amino acid sequences. B: scFv V_L translated amino acid sequences.
Figure 4.7. Jalview sequence alignment showing conservation and consensus of amino acid sequences among non-antibody scFv V\(_L\) clones. Sequence lengths (number of amino acids) are listed beside sequence names. Conservation numbers (0-9, 10 = *) provide numerical scale for degree of physiochemical conservation at any given amino acid position, as calculated by Livingstone and Barton (1993). Consensus letters refer to amino acid homology present in more than two sequences for a position; A “+” indicates there are two or more conflicting homologies for that position (Waterhouse et al., 2009). Amino acid sequence of scFv 6B1 V\(_L\) listed as bottom-most entry for comparison. Conservation and consensus information provided for non-antibody VL sequences only (“non-VL”), and for non-antibody VL sequences with 6B1 VL sequence for comparison.
After determining their sequences, each \( V_H/V_L \) gene insert pair was cloned into a pIT2 plasmid with a \((G_4S)_3\) linker in a \( V_H\)-linker-\( V_L \) orientation. Using the online ProtParam tool and the translated amino acid sequences, the molecular weights and several other protein characteristics of the scFvs were calculated (see Table 4.2). The calculated instability indices classified all 11 scFvs as ‘unstable’ (Gasteiger et al., 2005).

**Table 4.2. Summary of calculated scFv characteristics.** Using the online ProtParam tool and translated scFv sequences, the molecular weight (MW), theoretical isoelectric point (pI), extinction coefficient (\( \varepsilon \)), instability index (II) and grand average of hydropathy (GRAVY) were calculated for each scFv (Gasteiger et al., 2005).

<table>
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<tr>
<th>scFv</th>
<th>MW (Da)</th>
<th>Amino Acids</th>
<th>pI</th>
<th>( \varepsilon ) (M^-1 cm^-1)</th>
<th>Instability Index (II)</th>
<th>GRAVY</th>
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<tr>
<td>1D3</td>
<td>25412.0</td>
<td>240</td>
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<td>56380</td>
<td>47.77 (unstable)</td>
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<td>-0.338</td>
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4.3.6. scFv expression and purification in *E. coli* HB2151

All 11 scFv pIT2 constructs were cloned into *E. coli* HB2151 for expression. Growth of strains containing scFv 3B6 and 10A4 coding sequences could not be achieved. The remaining nine scFvs were expressed in *E. coli* HB2151 using an IPTG-inducible system. Cells were lysed using chicken-egg lysozyme and DNase I, and soluble lysate fractions were analysed by SDS-PAGE and immunoblot for scFv content. 6B1 was the only scFv showing a discernable expression band at the expected molecular weight of
~25 kDa when probed with mouse anti-c-myc antibody (see Figure 4.8) or mouse anti-6xHis antibody (results not shown).

The soluble fraction of an enzymatic cell lysate containing 6B1 was Protein L-purified and expression was analysed further by SDS-PAGE and immunoblot (Figure 4.9). Bands at the expected molecular weight of ~25 kDa were visible in all cell fractions analysed, including a Protein L-purified sample. Yield of purified scFv was approximately 0.5 mg/L cell culture.

6B1 was also IMAC-purified using denaturing conditions (8 M urea and cell disruption by sonication), in an attempt to increase yield of the purified scFv. SDS-PAGE and immunoblot analysis of soluble cell and purified fractions did not show any bands at the expected ~25 kDa molecular weight when probed with anti-c-myc antibody (see Figure 4.10) or mouse anti-6xHis antibody (results not shown). This indicated degradation of all antibody products during the purification process.

4.3.7. IC50s of scFv 6B1 with respect to NLPs

The affinity of scFv 6B1 binding to the NLPs was evaluated by CI-ELISA with crude cell extract and calculated as the half-maximal inhibitory concentration (IC50) for each NLP (see Figure 4.11). IC50s were calculated using a log(inhibitor) vs. response model \(Y=\text{Bottom} + \frac{\text{Top-\text{Bottom}}}{[1+10^{[[X-\text{LogIC50}]]}]}\) in the software program GraphPad Prism for Mac 5.00. Based on binding results from indirect ELISA (results not shown), CI-ELISAs were performed using enzymatic cell lysate containing 6B1 and were run a minimum of two times for each NLP; as such, a mean IC50 for each NLP was calculated from IC50s generated from individual CI-ELISAs (see Figure 4.11).
Figure 4.8. scFv crude expression profile in *E. coli* HB2151. scFvs were expressed in *E. coli* HB2151 using an IPTG-inducible system. Cells were lysed with chicken-egg lysozyme and DNase I and centrifuged to separate soluble and insoluble cellular material. Soluble lysate fractions were analysed by reducing Coomassie-stained SDS-PAGE (A) and anti-c-myc probed immunoblot (B). Lane 1: protein molecular weight standard; Lane 2, 1D3; Lane 3, 1H8; Lane 4, 3H7; Lane 5, 5A6; Lane 6, 6B1; Lane 7, 7D2; Lane 8, 7H4; Lane 9, 8E2; Lane 10, 9C8. Lysates were loaded neat in all wells.
Figure 4.9. scFv 6B1 expression profile in *E. coli* HB2151. scFv was expressed in *E. coli* HB2151 using an IPTG-inducible system. Cells were lysed with chicken-egg lysozyme and DNase I, and cell suspensions were centrifuged to separate soluble and insoluble cell fractions. Cellular fractions were analysed by reducing Coomassie-stained SDS-PAGE (A) and anti-c-myc probed immunoblot (B). Lane 1: protein molecular weight standard; Lane 2: cellular expression medium; Lane 3: insoluble cell fraction, resuspended in PBS; Lane 4: soluble cell fraction; Lane 5: Protein L-purified sample. All samples were loaded neat.
Figure 4.10. scFv 6B1 denaturing purification on IMAC FPLC. scFv was expressed in *E. coli* HB2151 using an IPTG-inducible system, solubilized in IMAC binding buffer containing 8 M urea and lysed via sonication. Cell suspension was centrifuged to separate soluble and insoluble cell fractions and soluble fraction was purified using IMAC on an FPLC. Soluble cell fraction and purified fractions were analysed by reducing Coomassie-stained SDS-PAGE (A) and anti-c-myc probed immunoblot (B). Lane 1: protein molecular weight standard; Lane 2: soluble cell fraction; Lane 3: flow through from column loading; Lane 4: purified fraction 1; Lane 5: purified fraction 2; Lane 6, purified fraction 3; Lane 7, purified fraction 4. Fractions were collected based on peak absorbance at 280nm (as measured by FPLC). All samples were loaded neat.
4.11. **IC50s of NLPs in relation to scFv 6B1 binding.** Microtitre plates were coated with 1.0 µM NLP and blocked using 1.5% casein in PBS. Soluble cell fractions containing 6B1 were pre-incubated with serial dilutions of the coated NLP before being applied to blocked plates. Plates were probed with mouse anti-c-myc-HRP secondary antibody and allowed to develop for 45 minutes using TMB substrate. Background was determined by applying PBS to coated, blocked wells in place of scFv. OD450 shown above represent means of background-subtracted triplicates ± SEM.
The IC50 for scFv 6B1 with NLP<sub>Bha</sub> was 66.6 nM; with NLP<sub>Fo</sub> it was 237 nM; with NLP<sub>Pya</sub> it was 39.9 nM; and with NLP<sub>Ssc1</sub> it was 188 nM.

4.3.8. Amino acid sequence of 6B1 IgG

6B1 V<sub>H</sub> and V<sub>L</sub> sequences were combined with human constant regions to reformat 6B1 from an scFv to a full-length chimeric IgG. The translated amino acid sequences for the IgG heavy and light chains are shown in Figure 4.12. CDRs are delineated for both V<sub>H</sub> and V<sub>L</sub> sequences, as are constant regions.

4.3.9. IgG expression and purification in <i>N. benthamiana</i>

6B1 IgG was expressed in <i>N. benthamiana</i> leaf tissue over the course of seven days, then extracted by mechanical homogenization in Protein G binding buffer. Total soluble protein (TSP) as measured by Bradford assay was 0.280 mg/mL crude plant extract; crude extract from <i>N. benthamiana</i> tissue infiltrated with p19 alone was 0.169 mg/mL. Reducing SDS-PAGE and immunoblot analysis confirmed the presence of 6B1 IgG in crude plant extract (see Figure 4.13.A and B), as evidenced by bands at 25 and 50 kDa; p19-only crude extract showed no 50 kDa band on SDS-PAGE, and no bands at 25 or 50 kDa on anti-human probed immunoblot. Protein G purification of 6B1 from crude plant extract was successful, also as evidenced by reducing anti-human probed immunoblot (see Figure 4.13.D); bands at 25 and 50 kDa were visible in three of four collected fractions. Based on A<sub>280</sub> readings of purified fractions, yield of purified IgG was calculated at 17.5 mg/kg plant tissue.
Figure 4.12. Translated sequence of 6B1 as chimeric IgG. scFv 6B1 V\textsubscript{L} and V\textsubscript{H} coding sequences were cloned into DNA cassettes containing human IgG constant regions for expression in \textit{N. benthamiana} plants as a full length IgG. Original 6B1 variable regions are highlighted in red; complementarity determining regions (CDR) are noted for both light (A) and heavy (B) chains. The \textit{Arabidopsis} basic chitinase signal sequence (ABC) precedes both V\textsubscript{L} and V\textsubscript{H} in order to facilitate expression in a plant system.
Figure 4.13. Expression profile of IgG 6B1 in *N. benthamiana*. IgG was expressed in *N. benthamiana* over a course of seven days. Plant matter was mechanically lysed with a Polytron blender, then filtered and centrifuged to soluble and insoluble fractions. Soluble fractions were analysed by reducing Coomassie-stained SDS-PAGE (A) and goat anti-human (Fab) probed immunoblot (B). Lane 1: protein molecular weight standard; Lane 2: soluble crude extract containing p19 only; Lane 3: soluble crude extract containing p19 and 6B1 IgG; Lane 4: Protein G-purified 6B1 IgG (diluted ½). Soluble *N. benthamiana* crude extract was purified on a Protein G column on FPLC. Purified fractions were analysed by reducing Coomassie-stained SDS-PAGE (C) and goat anti-human (H+L) probed immunoblot (D). Lane 1: protein molecular weight standard; Lane 2: purified fraction 1; Lane 3: purified fraction 2; Lane 4: purified fraction 3; Lane 5: purified fraction 4. Fractions were collected based on peak absorbance at 280 nm (as measured by FPLC). All samples were loaded neat.
4.3.10. NLP detection and binding with 6B1 IgG

6B1 IgG was tested for the ability to bind NLPs in immunoblot format. NLP\textsubscript{Bha}, NLP\textsubscript{Fo}, NLP\textsubscript{Sscl}, NLP\textsubscript{Pya} and commercial BSA were run on SDS-PAGE in equal amounts (2 µg each) and transferred to PVDF membrane. The membrane was probed with 6B1 IgG in a final concentration of 40 nM as primary antibody (see Figure 4.14). Bands at approximately 24 kDa corresponding to NLP\textsubscript{Bha}, NLP\textsubscript{Pya} and NLP\textsubscript{Sscl} were visible, as well as at a 25 kDa band corresponding to NLP\textsubscript{Fo}. A band corresponding to BSA (~66 kDa) was visible on the SDS-PAGE gel, but not on the 6B1-probed immunoblot.

Figure 4.14. Western blot analysis of 6B1 IgG binding to four NLPs.

Purified protein samples were run on reducing SDS-PAGE gels in amounts approximating 2 µg per well. Gels were run in duplicate then analyzed by Coomassie stain (A) and immunoblot probed with 40 nM 6B1 IgG and goat anti-human (Fab)-AP (B). Lane 1: protein molecular weight standard; Lane 2: purified NLP\textsubscript{Bha}; Lane 3: purified NLP\textsubscript{Fo}; Lane 4: purified NLP\textsubscript{Pya}; Lane 5: purified NLP\textsubscript{Sscl}; Lane 6: commercial BSA.
6B1 IgG was also tested for the ability to bind NLPs in indirect ELISA format using crude *N. benthamiana* leaf extract. OD$_{450}$ for crude extract containing 6B1 IgG was a minimum of three times background for all four NLPs; background was measured as OD$_{450}$ for *N. benthamiana* crude extract containing p19 only (results not shown).

4.3.11. Biacore analysis of 6B1 IgG

Surface plasmon resonance analysis of 6B1 IgG binding to NLPs was attempted using Biacore technology. Many different experimental conditions were tested (see Table 4.1), including various buffers, surface densities, antigen concentrations, coating with antibody followed by injecting NLP and coating with NLP followed by injecting antibody, but none were successful in showing any discernable difference in binding between 6B1 and a negative control IgG. An anti-human capture kit was employed to aid in correct orientation of 6B1 IgG Fabs for NLP binding. Binding to captured 6B1 was observed with NLP$_{Bha}$ (see Figure 4.15.A) but the dissociation was biphasic, indicating NLP$_{Bha}$ had aggregated in solution. Aggregation was confirmed though SEC analysis (Figure 4.15.B). Fresh monomeric NLP$_{Bha}$ was SEC purified and injected over a fresh capture-6B1 surface at concentrations ranging from 0.25 µM to 2.5 µM, but no discernable difference in binding between 6B1 and a negative control IgG was observed (Figure 4.15.C).
Figure 4.15. Biacore test injections for NLP$_{\text{Bha}}$. A Biacore T200 machine (GE Healthcare) and mouse anti-human IgG capture kit were employed to attempt to measure the capacity of 6B1 IgG to bind NLP$_{\text{Bha}}$. Mouse anti-human IgG was immobilized to the chip surface by amine coupling at a surface density of 11017 RU; 6B1 IgG was bound by the capture antibody (5400 RU, theoretical R$_{\text{max}}$~1800) and NLP$_{\text{Bha}}$ was injected on the chip surface. A, NLP$_{\text{Bha}}$ was injected at concentrations of 0.5 µM and 1.0 µM. Binding to captured 6B1 was observed but dissociation was biphasic. B, NLP$_{\text{Bha}}$ was SEC-analysed to confirm presence of aggregates. C, Fresh NLP$_{\text{Bha}}$ was purified by SEC and monomeric fractions were injected on a fresh capture-6B1 surface; no specific binding was seen.
4.3.12. IC50s of 6B1 IgG with respect to NLPs

The affinity of 6B1 IgG binding to the NLPs was evaluated by CI-ELISA and calculated as the half-maximal inhibitory concentration (IC50) for each NLP (see Figure 4.16). IC50s were calculated using a log(inhibitor) vs. response model 

\[ Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{\left[X - \log(\text{IC50})\right]}} \]

in the software program GraphPad Prism for Mac 5.00. CI-ELISAs were performed using Protein G-purified 6B1 IgG and were run a minimum of three times for each NLP; as such, a mean IC50 for each NLP was calculated from IC50 generated from individual CI-ELISAs (presented in Figure 4.16). The IC50 for 6B1 IgG with NLP\textsubscript{Sscl} was 71.3 nM; with NLP\textsubscript{Bha} was 103 nM; with NLP\textsubscript{Pya} was 124 nM; and with NLP\textsubscript{Fo} was 1258 nM (1.26 µM).

4.4. Discussion

Mice were immunized with NLP-heptapeptide GHRHDWE to raise antibodies that were specific and cross-reactive to several NLPs. Due to its extremely low molecular weight (936 Da), it was necessary to conjugate the NLP-heptapeptide to a larger carrier protein to elicit an immune response. The carrier protein keyhole limpet hemocyanin (KLH) was selected for this role, based on its high molecular weight (100 kDa) and its well-documented immunogenicity (Helling et al., 1994). Initial serum responses were measured from a test bleed taken 10 days after the second boost (51 days after the first immunization). IgG and IgM in the sera had high affinity for and high titres to the NLP-heptapeptide and minimal cross-reactivity to an unrelated peptide, indicating a robust antibody response was developing following immunizations and boosting. The IgM to IgG ratio measured in the first test bleed indicated good antibody class-switching from
in relation to IgG 6B1 binding. Microtitre plates were coated with varying concentrations of the respective NLP as listed below. Varying concentrations of 6B1 IgG (listed below) were pre-incubated with serial dilutions of the coated NLP before being applied to blocked plates. Plates were probed with goat anti-human (H+L)-HRP secondary antibody and allowed to develop for 45 minutes using TMB substrate. Background was determined by applying PBS to coated, blocked wells in place of IgG. NLP coat and IgG concentrations (respectively) were: A, NLP_{Bha} 0.25 \mu M and 6B1 8 nM; B, NLP_{Fo} 1.0 \mu M and 6B1 8 nM; C, NLP_{Pya} 1.0 \mu M and 6B1 8 nM; D, NLP_{SscI} 1.0 \mu M and 6B1 16 nM. OD_{450} shown above represent means of background-subtracted triplicates ± SEM.
the former to the latter. Indirect ELISA analysis of the first test bleed also suggested that the immune responses of all four mice were maturing IgG antibodies with suitable affinities to the GHRHDWE heptapeptide, as was expected. When the hybridoma clones were isotyped, however, it was discovered that all 70 stable secretors were producing IgM class antibodies. This was both unexpected and undesired. IgM antibodies have not undergone somatic hypermutation, meaning the antibodies being produced were not affinity matured in vivo and would likely be weak binders unless they were maintained in their secreted decameric forms (relying on avidity to compensate for lack of affinity). Secreted IgM molecules are much less practical to work with, both due to their size (900 kDa) and the lack of commercially available IgM-specific reagents. It would also be very difficult to assess binding to NLPs without being able to rely on a monomeric binding model.

Since all 70 hybridoma clones were found to be IgM by isotyping, it was decided that remaining mice 2 and 3 would receive two more boosts to favour antibody production before a second test bleed was performed. The IgM responses of the mice, however, remained unchanged (i.e., in the order of 25% to 35% of the IgG response). This indicated that there was no significant decrease in IgM response after the two extra boosts and that the additional immunizations did not appear to increase class switching to IgG antibodies. A second fusion with splenocytes from mice 2 and 3 was not pursued, as it was unlikely to produce any more stable IgG secretors than the first fusion. Instead, work with the existing IgM clones was pursued. Due to the unwieldy size of IgMs and the impracticality of producing them in vitro via hybridoma cell culture, it was decided to clone the V_H and V_L sequences of the highest binders to create scFvs.
All 70 IgM hybridoma supernatants were assessed for binding using an indirect ELISA. Most IgM clones showed good binding and cross-reactivity to at least one and in some cases all four NLPs. The top 24 binders were fragmented to create F(ab')\textsubscript{µ} antibodies to approximate the univalent binding of an scFv, and binding was again assessed by indirect ELISA. The 24 F(ab')\textsubscript{µ} clones showed very poor NLP binding compared to their IgM progenitors, likely due to degradation during fragmentation and elimination of the IgMs avidity factor. Nonetheless, the best 11 binders were chosen for cloning to create scFvs.

The nucleotide sequences for the V\textsubscript{H} and V\textsubscript{L} genes for all 11 clones were ascertained from sequencing data. A minimum of five gene inserts for each V\textsubscript{H} and V\textsubscript{L} sequence were sequenced, translated and compared for consensus and the presence of canonical antibody structures. Many of the translated V\textsubscript{L} sequences did not contain cysteine pairs or recognizable CDRs, thus necessitating repeat sequencing rounds of multiple clones. All scFv heavy chains showed different and diverse CDRs, particularly CDR3, indicating that the 11 scFvs analysed were distinct antibodies that bound to the same antigen. This contrasted heavily with the scFv light chain sequences, which showed very little diversity among CDRs and, in several cases, were identical among separate clones. CDR diversity results from productive somatic hypermutation, a process that occurs after antigen stimulation activates mature B cells. The diverse sequences of the analysed V\textsubscript{H} CDRs indicated that the somatic hypermutation process may have taken place for these gene sequences, as expected. Due to the prolific nature of somatic hypermutation, the probability of the process resulting in identical CDR sequences among separate clones is almost nonexistent. Thus, it is unlikely that the lack of diversity
among V\(_L\) sequences arose from productive somatic hypermutation. Rather, the similarity of the V\(_L\) sequences may be due to a partial or total lack of the somatic hypermutation process maturing these variable region genes.

The immune responses of the mice appear to have been arrested during the B cell activation process, resulting in antibodies of a single isotype and diminishing their final sequence diversity and range of affinities. Several factors may have contributed to this occurrence, though it is not clear which, if any, of them was significant enough to affect B cell activation and differentiation. Mice 1 and 4, the source of the splenocytes for hybridoma fusion, died of anaphylactic shock immediately after their final boost; thus, this final boost had little to no effect on the maturation of the mice immune responses. This, however, does not explain why the IgM titres of the remaining mice, i.e., mice 2 and 3, remained high after their third and fourth boosts. Somatic hypermutation occurs in germinal centres following stimulation with a thymus-dependent antigen, as does the process of class-switching from IgM to IgG, IgA and IgE isotypes; the absence of either process is characteristic of an immune response to a thymus-independent antigen. However, immunization with KLH produces a prototypical thymus-dependent immune response and typically includes both of these processes (Amlot et al., 1985). A more likely possibility is that the NLP-heptapeptide was too small an antigen to develop a fully mature humoral immune response, even when conjugated to KLH. The use of KLH as a carrier could have compounded this effect, as KLH immunizations tend to result in much higher IgM titres than immunizations with other common carriers like BSA or ovalbumin (Vidal and Rama, 1994; Helling et al., 1994; Lebrec et al., 2013).
Using the online ProtParam tool and the translated amino acid sequences, an instability index (II) was calculated for each scFv. An II of 40 or greater indicates that the protein is predicted to be unstable in a test tube (Gasteiger et al., 2005). Using this definition, all 11 scFvs would be classified as unstable, the least unstable being 6B1 with an II of 40.51. This could explain why 6B1 was the only scFv to show any discernable expression in *E. coli* HB2151. Instability is not uncommon among scFvs, and is widely regarded as the main challenge in their engineering and the main obstacle in their expression and purification (Borras et al., 2010). This instability would also account for the apparently poor expression levels (0.5 mg/L *E. coli* culture) observed for 6B1, as most scFv was likely produced in inclusion bodies. Low yield was compounded by precipitation after purifying (results not shown). Binding to NLPs could be quantified in CI-ELISA format by testing 6B1 in crude *E. coli* cell lysate, but this binding was not reproducible in purified samples of 6B1. IMAC purification of 6B1 using denaturing conditions (8 M urea) was attempted, but no scFv was detectable in any of the analysed fractions. Due to its poor yield and instability, it was concluded that 6B1 was not a viable research tool in its scFv form.

Since 6B1 was the only anti-NLP antibody to demonstrate enough expression for binding evaluation, the decision was made to study it by reformatting it to create a stable IgG. The 6B1 V<sub>H</sub> and V<sub>L</sub> regions raised in mouse were combined with human κ and G1 constant regions to create a chimeric anti-NLP IgG. 6B1 IgG was successfully expressed in and purified from *N. benthamiana* leaf tissue with a final yield of 17.5 mg purified antibody per kg plant material. This was a 35-fold increase from the yield of purified 6B1
as an scFv expressed in *E. coli* HB2151. 6B1 IgG also had much greater stability than its scFv form, in that it did not precipitate after purification, even at high concentrations.

6B1 IgG binding to NLPs was confirmed by immunoblot analysis. Bands corresponding to all four NLPs were visible, while a band corresponding to a BSA negative control was not. This indicated that 6B1 IgG bound all four NLPs specifically, without cross-reaction to unrelated BSA. Unlike anti-NLP<sub>pya</sub> rabbit serum, binding of NLP<sub>Fo</sub> by 6B1 IgG was detected by immunoblot. Based on the relative intensity of the bands, 6B1 IgG affinity appeared to be highest to NLP<sub>Bha</sub> and NLP<sub>pya</sub>, then NLP<sub>Ssc</sub> and lowest to NLP<sub>Fo</sub>.

Indirect ELISA was used to compare relative NLP binding by 6B1 in four antibody formats: IgM (in TC supernatant), F(ab’)<sub>µ</sub> (fragmented from IgM), scFv (in *E. coli* HB2151 enzymatic cell lysate) and IgG (in *N. benthamiana* crude extract); these data are summarized in Figure 4.17. F(ab’)<sub>µ</sub>, scFv and IgG all showed similar relative binding to the NLPs. The F(ab’)<sub>µ</sub> showed the poorest binding to the NLPs, which may have been compounded by this antibody experiencing degradation during the fragmentation process. Poor binding was also observed by the scFv, which was of low concentration in *E. coli* lysate and known to be unstable, as discussed above. The IgG showed better binding than the scFv, but only marginally. This was expected, as the only major changes between the IgG and scFv formats was the addition of constant regions and a switch from mono- to divalency. What was surprising was the vast overall difference in binding strength between the IgM and its F(ab’)<sub>µ</sub>, scFv and IgG counterparts, whose OD<sub>450</sub>’s were ten to twenty times less. While varying concentrations can partially explain the discrepancy, the major difference appears to be that IgMs are decavalent, while IgGs are divalent and
F(\text{ab}')\mu s and scFvs are monovalent. Normally the \textit{in vivo} antibody maturation process accounts for this variation by replacing ten weak IgM binding sites with two strong IgG binding sites. 6B1 likely did not undergo affinity maturation \textit{in vivo}, so the affinity increase associated with matured paratopes is not a factor. Thus, avidity must account for the differences observed: the IgM has ‘better’ binding because it has ten weak binding sites, while the IgG has two weak binding sites and the F(\text{ab}')\mu and scFv have only one.

As with rabbit anti-NLP\textsubscript{Pya} serum, all four formats of 6B1 showed poorest binding to NLP\textsubscript{F0} (relative to the other NLPs tested). This difference in binding may be due to reduced exposure of the NLP-heptapeptide on the protein surface of NLP\textsubscript{F0} (as discussed in Chapter 3). Also, like the rabbit serum, 6B1 IgM showed the strongest binding to NLP\textsubscript{Bha}, followed by NLP\textsubscript{Ssc1} and NLP\textsubscript{Pya}. 6B1 scFv and IgG did not show much difference in binding to NLP\textsubscript{Bha}, NLP\textsubscript{Pya} and NLP\textsubscript{Ssc1}.

Analysis of 6B1 IgG binding to the NLPs was attempted through surface plasmon resonance using Biacore technology but binding could not be shown using this method. Attempts with 6B1 immobilized were not successful, despite troubleshooting with numerous experimental conditions (as listed in Table 4.1). Immobilization of NLPs was also attempted but the low pH used for immobilization and/or the harsh conditions during the amine coupling process seemed to destroy the proteins’ activity, since the experimental R\textsubscript{max} of the surfaces was less than 1%. Some binding was observed between anti-human-captured 6B1 and NLP\textsubscript{Bha}; however, the dissociation was biphasic, indicating that NLP\textsubscript{Bha} had aggregated in solution. This fact was confirmed using SEC. Binding to fresh monomeric NLP\textsubscript{Bha} was not observed, even at high concentrations. It was concluded that Biacore analysis of 6B1 binding was not possible because the NLPs aggregated.
Figure 4.17. Summary of 6B1 binding to NLPs in different antibody formats. 6B1 binding to NLPs was tested in indirect ELISA format by coating NLPs at 1 μM and applying 6B1 as primary antibody in the following formats: IgM in TC supernatant (diluted 1/10); F(ab')2 fragmented from IgM (diluted 1/10); scFv in *E. coli* HB2151 enzymatic cell lysate (neat); IgG in *N. benthamiana* crude extract (neat). OD_{450} pictured above are means of background-subtracted duplicates (in the case of IgM and F(ab')2) or triplicates (in the case of scFv and IgG).
Quantification of 6B1 binding was accomplished through CI-ELISA for both the scFv and IgG forms. As scFv 6B1 was not stable when purified, its binding was assessed using *E. coli* HB2151 cell lysates containing unpurified scFv. The IC50 data for both antibody forms, as well as for the anti-NLP<sub>Pya</sub> rabbit serum, are summarized in Table 4.3. As with anti-NLP<sub>Pya</sub> rabbit serum, NLP<sub>Fo</sub> had the highest IC50 of all four NLPs for 6B1 scFv. The scFv IC50 for NLP<sub>Fo</sub> was 238 nM, three times lower than its IC50 with rabbit serum (669 nM). Unlike anti-NLP<sub>Pya</sub> rabbit serum, binding of scFv 6B1 to NLP<sub>Bha</sub> and NLP<sub>Sscl</sub> were quite different. NLP<sub>Bha</sub> had the second lowest IC50 at 66.6 nM, while NLP<sub>Sscl</sub> had the second highest at 188 nM; i.e., the binding affinity of rabbit serum was reduced by six and 16 times, respectively. scFv 6B1 had the highest affinity for NLP<sub>Pya</sub>, with an IC50 of 39.9 nM, three times higher than its rabbit serum counterpart (121 nM). Thus, the IC50s of the scFv 6B1 were higher than their rabbit serum counterparts for NLP<sub>Bha</sub> and NLP<sub>Sscl</sub>, but lower for NLP<sub>Fo</sub> and NLP<sub>Pya</sub>. The lowest scFv IC50 was 39.9 nM (for NLP<sub>Pya</sub>), four times greater than the lowest rabbit serum IC50 of 10.5 nM (for NLP<sub>Bha</sub>). When comparing rabbit serum binding to NLPs it was found that the highest IC50 (for NLP<sub>Fo</sub>) was 64 times greater than the lowest (for NLP<sub>Bha</sub>), a gap of nearly two orders of magnitude. For scFv binding to the NLPs, the highest IC50 (NLP<sub>Fo</sub>) was only six times greater than the lowest NLP (NLP<sub>Pya</sub>), meaning the gap between them was less than one order of magnitude. Thus, the affinity of 6B1 scFv for all four NLPs was similar and may be due to 6B1 being raised from a molecular target common to all four proteins, i.e., the NLP-heptapeptide. It is notable that, although 6B1 scFv showed similar binding affinity among all antigens tested, its range of affinity was diminished compared to that of the anti-NLP<sub>Pya</sub> rabbit serum, that is, the increase in cross-reactivity and specificity of
scFv 6B1 to some NLP antigens (namely NLP_{Fo}) came at the sacrifice of affinity to others (NLP_{Bha} and NLP_{Ssc}).

In its IgG format, 6B1 had higher IC50s than rabbit serum for all four NLPs. The highest IgG IC50 (i.e., to NLP_{Fo}) was twice that of its rabbit serum counterpart. The lowest IC50 (i.e., to NLP_{Ssc}) of the IgG was almost seven times greater than was the lowest rabbit serum IC50 (to NLP_{Bha}). After reformatting from an scFv to an IgG, the IC50s of 6B1 to NLP_{Bha}, NLP_{Fo} and NLP_{Pya} all increased: IC50 to NLP_{Bha} increased from 66.6 nM to 103 nM; IC50 to NLP_{Fo} from 238 nM to 1258 nM (or 1.258 \mu M); and IC50 to NLP_{Pya} from 39.9 nM to 124 nM. Interestingly, the IC50 increase for NLP_{Pya} brought it back in line with its rabbit serum IC50 of 121 nM. For NLP_{Fo} and NLP_{Bha}, however, the increases meant their IgG IC50s were two and ten times their rabbit serum counterparts, respectively. The only NLP to which 6B1 showed a decrease in IC50 was NLP_{Ssc} at 71.3 nM, less than half that of its scFv progenitor. This was unexpected, given that the reformat from scFv to IgG diminished its binding capacity for the other NLPs. Since NLP_{Ssc} was found to be a dimer in solution, the increased ability of 6B1 IgG to bind NLP_{Ssc} could be the result of an avidity effect between what are, essentially, two multivalent protein complexes. This phenomenon would only be seen with 6B1 IgG, as its scFv form is monovalent. Avidity with the NLPs is unlikely in their monomeric forms, as the 6B1 epitope (the NLP-heptapeptide) is located inside a small pocket on the protein surface. This pocket is thought to coordinate a calcium ion (Ottmann et al., 2009), and thus would not be large enough for two 6B1 paratopes to bind simultaneously to a single NLP molecule. For IgG binding to the NLPs, the highest IC50 (to NLP_{Fo}) was 16 times higher than the lowest (to NLP_{Ssc}), meaning the gap between them was approximately
one order of magnitude. Even though the affinity of 6B1 IgG to the NLPs was diminished compared to its scFv form (with the exception of binding to NLP$_{Ssc}$), the overall increase in cross-reactivity gained by raising a monoclonal antibody against a conserved structure was maintained during the switch from scFv to IgG format.

Table 4.3. Summary of IC50s with respect to NLPs. IC50s for NLP$_{Bha}$, NLP$_{Fo}$, NLP$_{Pya}$ and NLP$_{Ssc}$ in relation to binding by polyclonal rabbit anti-NLP$_{Pya}$ serum, monoclonal scFv 6B1, and monoclonal IgG 6B1. Data were determined by CI-ELISA, as described previously in 3.2.7, 4.2.13.1 and 4.2.13.2 and presented in Figures 3.6, 4.11 and 4.16. All IC50s are listed in nM quantities.

<table>
<thead>
<tr>
<th></th>
<th>NLP$_{Bha}$</th>
<th>NLP$_{Fo}$</th>
<th>NLP$_{Pya}$</th>
<th>NLP$_{Ssc}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit α-NLP$_{Pya}$ Serum (pAb)</td>
<td>10.5</td>
<td>669</td>
<td>121</td>
<td>11.3</td>
</tr>
<tr>
<td>6B1 α-heptapeptide scFv (mAb)</td>
<td>66.6</td>
<td>238</td>
<td>39.9</td>
<td>188</td>
</tr>
<tr>
<td>6B1 α-heptapeptide IgG (mAb)</td>
<td>103</td>
<td>1258</td>
<td>124</td>
<td>71.3</td>
</tr>
</tbody>
</table>

The experiments detailed in this chapter provide more than just the means to produce a molecular tool. What ultimately resulted was a comparison of the cross-reactivity of a single antibody presented in multiple formats, with several antigens of substantially different primary structure but with one similar epitope. It was found that in the absence of somatic hypermutation, IgM was by far the most efficient antibody format as it allows avidity to compensate for a lack of affinity. It was also determined that despite high affinity and cross-reactivity, the instability of an scFv can render it impractical as an experimental tool. Finally, it was shown that the challenges of producing an unstable scFv can be circumvented by reformatting to an IgG, without significant loss of binding or cross-reactivity.
The goal of this chapter was to raise a monoclonal antibody that specifically recognized multiple NLPs. Despite several setbacks, this goal was ultimately met. A chimeric IgG was produced that cross reacts to four NLPs in a quantifiable manner. The purpose of raising an anti-NLP antibody was not just to detect NLPs, but also to neutralize their effects on plants. This function was evaluated in Chapter 5.
5. **Effect of Native and Neutralized Nep1-Like Proteins on Seed Germination, Plant Development and Tissue Health**

5.1. Introduction

The majority of neutralizing antibodies have been developed in a clinical context to mitigate or treat human afflictions. One of the earliest uses of neutralizing antibodies was the treatment of poisonous snake bites with animal antisera (Bortnik and Rozanov, 1979). This practice has evolved to the engineering of monoclonal antibodies capable of neutralizing specific toxins like the shiga toxins produced by *E. coli* O157:H7 (Tzipori et al., 2004). Neutralizing antibodies and vaccines that elicit neutralizing antibodies are also being developed as treatments for viruses like HIV-1 (Kwong et al., 2011) and Hepatitis C (Di Lorenzo et al., 2011). While many antibodies exist against antigens with non-medical significance, their use is mainly limited to detection and quantification rather than neutralization. The use of neutralizing antibodies in a non-medicinal context is a relatively unexplored area of antibody application.

Select NLPs have been evaluated previously through PVX expression in *N. benthamiana* plants to determine their relative strength as elicitors of necrosis. Using this method, NLP_{Bha} and NLP_{F0} were both found to be relatively weak elicitors of necrosis compared to their *Phytophthora sojae* counterpart (Qutob et al., 2002). The effects of heterologously expressed NLP_{Bc1} and NLP_{Bc2} (a.k.a. BcNEP1 and BcNEP2) from *Botrytis cineria* have also been studied through direct infiltration in *N. benthamiana* leaves (Schouten et al., 2008). Thus, infiltration in plant leaves has been found to be an effective method to compare the relative necrotic abilities of the NLPs. Whole-plant studies of NLP activity are much less common and do not typically involve isolated NLP,
as these would involve infiltration of exogenous protein or binary vectors containing protein coding sequences to entire plant structures. Studies on seeds are also uncommon, as the assays involving seeds do not generate the qualitative results observed from leaf infiltrations. An ability to inhibit germination of *Nicotiana tabacum* seeds has been observed with NLP_{Fo}, though a threshold limit was not determined (Meir et al., 2009). To the best knowledge of this author, no detailed study of the effect of exogenous NLP application on seed germination and seedling development has yet been published.

Chapter 3 described the development of a polyclonal antibody assay based on the ability of serum raised against one NLP, NLP_{Pya}, to bind multiple NLPs. Chapter 4 expanded on this idea by raising a monoclonal antibody specific to the conserved NLP-heptapeptide for the purpose of cross-reacting to multiple NLPs. This chapter explores the applicable functions of both reagents by assessing their NLP neutralization capabilities through spot infiltration experiments. The ability of the polyclonal assay to detect and quantify NLP_{Pya} from a hydroponic *P. aphanidermatum* infection was also evaluated. While examining these antibody applications, the aforementioned experiments also revealed several aspects of the relationship between NLP_{Pya} and its roles in plant disease and pathogenicity. It was found that NLP_{Pya} was capable of inhibiting seed germination and seedling development when supplemented in plant growth medium. The symptom of root browning in diseased plants, a key indicator of the pathogen transition from biotrophy to necrotrophy (Sutton et al., 2006), was also found to coincide with the production of NLP_{Pya} during *P. aphanidermatum* infection.
5.2. Methods and Materials

5.2.1. MS-NLP$_{\text{Py}}$ media preparation and seed planting

NLP$_{\text{Py}}$ was expressed in *E. coli* BL21 (DE3) and IMAC-purified under denaturing conditions as described in section 3.2.2 and checked for purity by SDS-PAGE and anti-6xHis probed immunoblot as described in section 3.2.5. Purified fractions were filter sterilized using 0.22 µm nitrocellulose syringe filters (Fisher Scientific) and quantified by spectrophotometric readings at 280 nm. *N. tabacum* seeds were surface-disinfected by submersion in 70% ethanol for one minute, then submersion in 0.5% bleach solution for ten minutes. Seeds were rinsed three times with distilled H$_2$O after each step. Murashige Skoog Basal Medium (MS) plus 2% agar was prepared and sterilized according to the manufacturer’s instructions (Sigma Aldrich). Filter-sterilized NLP$_{\text{Py}}$ was diluted in cooled molten MS to final concentrations of 0.2 µM, 1.0 µM and 5.0 µM. NLP$_{\text{Py}}$-amended growth medium (MS-NLP$_{\text{Py}}$) was poured into sterile 10 cm Petri plates (Fisher Scientific) at 20 mL per plate; three plates per NLP$_{\text{Py}}$ concentration were prepared, plus three plates of unamended MS.

Growth medium was cooled at room temperature until solidified, then disinfected *N. tabacum* seeds were planted on the media surface. Six seeds per plate were planted, for a total of 18 seeds per treatment times four treatments. NLP$_{\text{Py}}$ sterilization, seed disinfection, media preparation and seed planting all took place in a laminar flow hood. To prevent contamination, plates were sealed with parafilm (Fisher Scientific) after seeds were planted. Seed planting took place on Day 0. Plates were incubated for 14 days post-planting at 20°C with an artificial light cycle of 16 h day/8 h night using a light intensity of 30-50 µE/s/m$^2$. Seedlings were harvested from MS-NLP$_{\text{Py}}$ medium on Day 14.
5.2.2. Seedling harvest from MS-NLP\textsubscript{p}ya media

Seedlings were harvested 15 days after planting. Seedling root lengths were measured immediately prior to harvest. In the case of seedlings grown in MSØ, primary, secondary and tertiary root lengths were measured and totalled per plant. Plants were extracted from the plates and excess growth medium was removed with sterilized forceps. Individual plants were placed in sterile 10 mL screw-cap glass containers and then inside a freeze dryer receptacle. Plants were freeze-dried at -50°C over the course of 24 h. Individual plants were then weighed using an analytical balance.

5.2.3. Spot infiltrations of \textit{N. benthamiana} leaf tissue

5.2.3.1. Inoculation with NLPs

Leaf tissue from six-week-old \textit{N. benthamiana} ΔFX plants was spot infiltrated with NLPs. NLP\textsubscript{Bha}, NLP\textsubscript{Fo}, NLP\textsubscript{p}ya and NLP\textsubscript{Sse} were diluted in PBS to concentrations of 0.25 µM, 0.5 µM, 1.0 µM, 2.0 µM, 4.0 µM and 8.0 µM. Using a needleless syringe (ResTek), spots of 10 µL were infiltrated by placing the syringe on the underside of the leaf tissue and slowly pressing the syringe plunger until the entire 10 µL volume was delivered into the leaf tissue. A minimum of three spots (replicates) per NLP dilution were infiltrated, as well as PBS, and syringes were changed between each dilution. As multiple plants were infiltrated, great care was taken to infiltrate leaves of approximately the same age/growth stage among plants. Plants were grown in a greenhouse with a natural light/dark cycle prior to infiltration and for two days following infiltration.
5.2.3.2. Inoculation with NLPs and anti-NLP<sub>Pya</sub> rabbit serum

Leaf tissue from six-week-old <i>N. benthamiana</i> ΔFX plants was spot infiltrated as described in 5.2.3.1, with the following changes. NLP<sub>Bha</sub>, NLP<sub>Fo</sub>, or NLP<sub>Pya</sub> were diluted in PBS to concentrations of 1.0 μM, 2.0 μM, and 4.0 μM. NLP dilutions were then mixed in equal volumes with PBS or with anti-NLP<sub>Pya</sub> rabbit serum (neat). Thus, final concentrations of each NLP were 0.5 μM, 1.0 μM and 2.0 μM, with or without anti-NLP<sub>Pya</sub> rabbit serum. NLPs were also mixed with rabbit preimmune serum (neat) to a final concentration of 0.5 μM NLP. All NLP dilutions were incubated at room temperature for 1 h prior to infiltration. A minimum of four spots (replicates) per NLP dilution were infiltrated, as well as PBS, anti-NLP<sub>Pya</sub> serum diluted ½ in PBS and rabbit preimmune serum diluted ½ in PBS. Plants were grown in a greenhouse with a natural light/dark cycle for four days after infiltration.

5.2.3.3. Inoculation with NLPs and 6B1 IgG

Leaf tissue from five-week-old <i>N. benthamiana</i> ΔFX plants was spot infiltrated as described in 5.2.3.1, with the following changes. NLP<sub>Bha</sub>, NLP<sub>Fo</sub>, or NLP<sub>Pya</sub> were diluted in PBS to concentrations of 2.0 μM. 6B1 IgG was diluted in PBS to concentrations of 0.5 μM or 1.0 μM. NLP dilutions were then mixed in equal volumes with PBS or with 6B1 IgG at either concentration. Thus, final concentrations of NLPs were 1.0 μM, with or without 6B1 IgG; 6B1 IgG concentrations were 0.25 μM or 0.5 μM. A minimum of three spots (replicates) per treatment were infiltrated, as well as PBS, and 6B1 IgG diluted to 0.25 μM and 0.5 μM in PBS. Plants were grown in a greenhouse with a natural light/dark cycle for four days after infiltration.
5.2.4. *P. aphanidermatum* infection of hydroponic *C. annuum* plants

Thiram-treated *Capsicum annuum* 35-206 RZ F-1 seeds were planted in rock wool and grown for ten days at 29°C, then 25 days at 24°C. After 35 days, individual *C. annuum* plants were transferred to single-plant 475 mL hydroponic units and grown using 0.73 g/L 7:11:27 N:P:K plus 0.48 g/L Ca(NO$_3$)$_2$ plant nutrient solution, at pH 5.8 (adjusted with KOH) and dissolved oxygen content provided by aeration. *P. aphanidermatum* P6 zoospores were prepared by inoculating a V8-juice agar plate and incubating for 48 h at 25°C, then cutting the colonized agar into 1 cm strips and immersing each strip in a Petri dish of distilled H$_2$O under fluorescent lights at 25°C for 48 h. Plates were then incubated under fluorescent lights at 20°C for 4 h to stimulate zoospore release. Aliquots (1 mL) of zoospores were vortexed, and immobilized zoospores were enumerated using a haemocytometer, and then diluted with plant nutrient solution to a concentration of 5 x 10$^3$ zoospores/mL plant nutrient solution. 8-week-old *C. annuum* plants were inoculated with *P. aphanidermatum* P6 by immersing their roots in the zoospore suspension (Day 0). Plants were incubated at 24°C with a relative humidity of 40%. Plant nutrient solution was sampled from *P. aphanidermatum*-inoculated plants and uninoculated plants on Day 0 and each of eight days following inoculation. Samples were stored at -20°C.

5.2.5. CI-ELISA analysis of *P. aphanidermatum*-infected solutions

Microtitre wells were coated for 1 h at 37°C with 100 µL of 1 µM NLP$_{Py}$ in PBS. After decanting and washing, wells were blocked with 200 µL 1.5% casein in PBS overnight at 4°C. To prepare a standard curve, NLP$_{Py}$ was serially diluted in PBS from
1.0 µM to 0.00049 µM (final concentrations). Rabbit anti-NLP<sub>Pya</sub> serum diluted 1/20 000 in PBS (final concentration) was mixed with NLP<sub>Pya</sub> serial dilutions and with <i>P. aphanidermatum</i>-infected solutions sampled from the experiment described in section 5.2.4. All mixtures were preincubated for one hour at room temperature in microcentrifuge tubes, then added to plate wells and incubated for one hour at room temperature. Goat anti-rabbit-HRP diluted 1/6000 in PBS was added to the wells and incubated for one hour at room temperature. Plates were developed using two-component TMB substrate (KPL), quenched using 0.1 M H<sub>2</sub>SO<sub>4</sub> after 30 minutes, then read at 450 nm. All reagents and samples were applied in triplicates of 100 µL unless otherwise stated; plates were washed five times between each step by pipetting 300 µL PBST into each well followed by decanting. CI-ELISAs were performed a minimum of three times for each <i>P. aphanidermatum</i>-infected plant being analysed, on different days and using freshly diluted reagents. Standard curves were generated from known NLP<sub>Pya</sub> concentrations (one per CI-ELISA run) and unknown NLP<sub>Pya</sub> concentrations were interpolated from their OD<sub>450</sub> readings using the GraphPad Prism 5.00 program for Mac.

### 5.3. Results

5.3.1. NLP<sub>Pya</sub> dose-response effect on <i>N. tabacum</i> seedling development

<i>N. tabacum</i> seeds were germinated and grown in the presence of various concentrations of NLP<sub>Pya</sub> for 15 days total. There were 18 seeds planted per treatment. Germination rates were: 16 seeds germinated on MSØ; 15 on 0.2 µM NLP<sub>Pya</sub>; 16 on 1.0 µM NLP<sub>Pya</sub>; and 10 on 5.0 µM NLP<sub>Pya</sub>. Single-factor ANOVA analysis of germination with a 99% confidence interval showed that there was a difference between the number
of seeds that germinated on 5.0 µM NLP<sub>Pya</sub> and those that germinated on MS∅, and MS∅ amended with 0.2 µM NLP<sub>Pya</sub> and 1.0 µM NLP<sub>Pya</sub> (p-value ≤ 0.0019). Seedlings germinated on MS∅ developed primary, secondary and tertiary leaf structures. Seedlings germinated on 0.2 µM and 1.0 µM NLP<sub>Pya</sub> developed primary and secondary leaves, while seedlings germinated on 5.0 µM NLP<sub>Pya</sub> developed primary leaves only (see Figure 5.1). Seedlings germinated on MS∅ developed primary, secondary and tertiary root structures, as well as root hairs, while seedlings germinated on 0.2 µM, 1.0 µM and 5.0 µM NLP<sub>Pya</sub> developed a primary root only, and no root hair (see Figure 5.2).

Mean root length for seedlings grown on MS∅ (totalled for primary, secondary and tertiary roots) was 23.5 ± 6.56 mm. Mean primary root length was 8.1 ± 1.45 mm, 10.1 ± 2.47 mm, and 2.7 ± 1.95 mm for seedlings grown on 0.2 µM, 1.0 µM and 5.0 µM NLP<sub>Pya</sub>, respectively (see Figure 5.3). The differences in mean root lengths were deemed statistically significant by single-factor ANOVA analysis with a 99% confidence interval (with a resulting p-value of <0.0001). Using Tukey’s multiple comparison test with a confidence interval of 99%, differences between all pairs of groups were also deemed statistically significant except for the differences between root lengths of seedlings grown on 0.2 µM NLP<sub>Pya</sub> compared to seedlings grown on 1.0 µM or 5.0 µM NLP<sub>Pya</sub>.

Mean dry weight for seedlings grown on MS∅ was 5.32 ± 1.26 mg. Mean dry weight was 0.83 ± 0.23 mg, 0.55 ± 0.16 mg, and 0.29 ± 0.22 mg for seedlings grown on 0.2 µM, 1.0 µM, and 5.0 µM NLP<sub>Pya</sub>, respectively (see Figure 5.4). Differences in mean dry weights were deemed statistically significant by single-factor ANOVA analysis (with a resulting p-value of <0.0001). Using Tukey’s multiple comparison test with a
Figure 5.1. *N. tabacum* seedling growth on various concentrations of NLP<sub>Pya</sub>. *N. tabacum* seeds were germinated and grown on Murashige Skoog plant growth medium amended with various concentrations of NLP<sub>Pya</sub>: A, no NLP<sub>Pya</sub> (MS∅); B, 0.2 µM NLP<sub>Pya</sub>; C, 1.0 µM NLP<sub>Pya</sub>; D, 5.0 µM NLP<sub>Pya</sub>. Time course of experiment from planting (Day 0) to harvest (Day 14) was 15 days total. Photos taken on Day 14 prior to seedling harvest.
Figure 5.2. Typical *N. tabacum* seedling development on various concentrations of NLP<sub>Pya</sub>. *N. tabacum* seeds were germinated and grown on Murashige Skoog plant growth medium amended with various concentrations of NLP<sub>Pya</sub>: A, no NLP<sub>Pya</sub> (MS∅); B, 0.2 µM NLP<sub>Pya</sub>; C, 1.0 µM NLP<sub>Pya</sub>; D, 5.0 µM NLP<sub>Pya</sub>. Time course of experiment from planting (Day 0) to harvest (Day 14) was 15 days total. Photos taken on Day 14 prior to seedling harvest.
Figure 5.3. Mean root length of N. tabacum seedlings developed on various concentrations of NLP\textsubscript{Pya}. N. tabacum seeds were germinated and grown on Murashige Skoog plant growth medium amended with various concentrations of NLP\textsubscript{Pya}. Seedling root lengths were measured on Day 15 of experiment, just prior to harvest. Lengths shown above represent mean totals of primary, secondary and tertiary roots (where applicable) of 15 plants per treatment (or 10 for 5.0 µM NLP\textsubscript{Pya}) ± SEM.
Figure 5.4. Mean dry weight of *N. tabacum* seedlings developed on various concentrations of NLP<sub>Pya</sub>. *N. tabacum* seeds were germinated and grown on Murashige Skoog plant growth medium amended with various concentrations of NLP<sub>Pya</sub>. Seedlings were harvested on Day 15, freeze dried, and weighed individually. Weights shown above represent mean weights of 15 plants per treatment (or 10 for 5.0 µM NLP<sub>Pya</sub>) ± SEM.
confidence interval of 99%, differences between dry weights of seedlings grown on MS∅ were deemed statistically significant compared to seedlings grown on all three concentrations of NLP_{Pya}.

5.3.2. NLP-induced necrosis in *N. benthamiana* leaf tissue

*N. benthamiana* leaf tissue was infiltrated with various concentrations of NLP_{Bha}, NLP_{Fo}, NLP_{Pya} and NLP_{Sscl}, as well as PBS. Visible necroses were seen on the leaf tissue in response to infiltrations with NLP_{Bha}, NLP_{Fo} and NLP_{Pya} (see Figure 5.5). Areas infiltrated with these NLPs turned from green to brownish-yellow and became translucent, dry, brittle, and almost paper-like in texture. Necrotic spots were approximately circular and measured 15 to 20 mm in diameter, corresponding to the area of plant tissue infiltrated with 10 µL NLP. This extreme necrosis was seen in infiltrations of NLP_{Pya} at concentrations ranging from 0.25 µM to 4.0 µM, and in infiltrations of NLP_{Bha} and NLP_{Fo} at concentrations of 1.0 µM and higher. Lower concentrations of NLP_{Bha} and NLP_{Fo} (<1.0 µM) produced a more mottled effect, alternating between healthy green tissue and yellowed necrotic spots. No necrosis was seen on leaves infiltrated with NLP_{Sscl}, even when using undiluted stock (8.1 µM, results not shown). All infiltrations with NLP_{Sscl} were indistinguishable from infiltration with PBS (see Figure 5.5). Mechanical wounding from the syringe used in the infiltration process, visible in Figure 5.5 as a ring ~5 mm in diameter, was not classified as necrosis produced by infiltration with NLPs.
Figure 5.5. Typical *N. benthamiana* leaf tissue spot infiltrated with NLPs.
Sections of *N. benthamiana* leaf tissue were infiltrated with 10 µL of varying concentrations of NLPs (see above) using a needleless syringe. Each spot infiltration was performed three times per NLP per concentration. Plants were six-weeks-old at time of infiltration and were grown for two days between infiltration and photos. *PBS was infiltrated as a negative control, one spot per infiltrated leaf; all PBS-only spots were infiltrated at a working concentration of 1x. See bottom-left plate (bottom-most PBS treatment) for scale.
5.3.3. Neutralization of NLP-induced necrosis in *N. benthamiana* leaf tissue via co-infiltration with anti-NLP<sub>Pya</sub> rabbit serum

*N. benthamiana* leaf tissue was infiltrated with various concentrations of NLP<sub>Bha</sub>, NLP<sub>Fo</sub>, and NLP<sub>Pya</sub>, with or without anti-NLP<sub>Pya</sub> rabbit serum or preimmune rabbit serum. NLP<sub>Ssc</sub> was not included because it did not cause necrosis. The necrotic spots caused by NLP<sub>Bha</sub>, NLP<sub>Fo</sub> and NLP<sub>Pya</sub> at concentrations of 0.5 µM, 1.0 µM and 2.0 µM were reproduced (see Figure 5.6). NLP<sub>Bha</sub> again showed mottled necrotic spots at lower concentrations (0.5 µM, 1.0 µM) and complete necrosis at a higher concentration (2.0 µM). Spots co-infiltrated with NLP<sub>Bha</sub> and anti-NLP<sub>Pya</sub> rabbit serum caused no necrosis at any of the concentrations tested. The same effect was seen with NLP<sub>Pya</sub> in that it caused consistent necrosis when infiltrated alone, but NLP<sub>Pya</sub> at these same concentrations showed no necrosis when it was co-infiltrated with anti-NLP<sub>Pya</sub> rabbit serum. Necrosis was seen for NLP<sub>Fo</sub> whether it was infiltrated alone or co-infiltrated with anti-NLP<sub>Pya</sub> rabbit serum. When infiltrated alone, anti-NLP<sub>Pya</sub> rabbit serum did not result in necrosis; neither did PBS or pre-immune rabbit serum. However, co-infiltrations of pre-immune rabbit serum with low concentrations of each NLP did result in necrosis. Mechanical wounding from the needleless syringe used was not classified as necrosis produced by infiltration with NLPs.
Figure 5.6. Typical *N. benthamiana* leaf tissue co-infiltrated with NLPs and anti-NLP<sub>Py</sub> serum. Six-week-old *N. benthamiana* leaf tissue was infiltrated with 20 µL NLP with or without rabbit anti-NLP<sub>Py</sub> serum (A). PBS, anti-NLP<sub>Py</sub> serum, rabbit pre-immune serum, and NLP plus rabbit pre-immune serum were also infiltrated (B). All sera were diluted to final concentrations of ½. Treatments were mixed and incubated one hour prior to infiltration. Four spots per treatment were infiltrated. Plants were grown for four days between infiltration and photos. See bottom-left plate (2.0 µM NLP<sub>B</sub> + anti-NLP<sub>Py</sub> serum treatment) for scale.
5.3.4. Neutralization of NLP-induced necrosis in *N. benthamiana* leaf tissue via co-infiltration with anti-NLP IgG

*N. benthamiana* leaf tissue was infiltrated with 1.0 µM concentrations of NLP$_{\text{Bha}}$, NLP$_{\text{Fo}}$, and NLP$_{\text{Pya}}$, with or without 6B1 IgG. Due to its lack of necrotic ability NLP$_{\text{Ssc1}}$ was once again excluded. As seen previously, infiltration with NLP$_{\text{Bha}}$, NLP$_{\text{Fo}}$ and NLP$_{\text{Pya}}$ produced necrotic spots (see Figure 5.7). When co-infiltrated with 0.25 µM 6B1 IgG, NLP$_{\text{Bha}}$ necrosis was reduced. Co-infiltration with 0.5 µM 6B1 IgG resulted in elimination of necrosis caused by NLP$_{\text{Bha}}$. When NLP$_{\text{Pya}}$ was co-infiltrated with 0.25 µM 6B1 IgG, the necrosis produced was mottled but reduced when compared to the NLP$_{\text{Pya}}$ only treatment. When co-infiltrated with 0.5 µM 6B1 IgG, NLP$_{\text{Pya}}$-induced necrosis was eliminated. Regardless of whether 0.25 µM 6B1 IgG was present, NLP$_{\text{Fo}}$ produced the same level of necrosis. When co-infiltrated with 0.5 µM 6B1 IgG, the necrotic spot produced was more mottled around the edges but still yellow-brown and brittle in the centre (at the injection site). 6B1 IgG was indistinguishable from PBS when infiltrated at 0.25 µM or 0.5 µM. Again, mechanical wounding was not classified as necrosis produced by infiltration with NLPs.
Figure 5.7. Typical *N. benthamiana* leaf tissue co-infiltrated with NLPs and 6B1 IgG. Five-week-old *N. benthamiana* leaf tissue was infiltrated with 20 µL of 1 µM of one of three NLPs, with or without 6B1 IgG at a final concentration of 0.25 µM or 0.50 µM (A). PBS and 6B1 IgG at concentrations of 0.25 µM and 0.5 µM were also infiltrated (B). Treatments were mixed and incubated one hour prior to infiltration. Each spot infiltration was performed three times per treatment using a 1mL syringe. Plants were grown for four days between infiltration and photos. See bottom-left plate (1.0 µM NLP$_{Bha}$ + 0.5 µM 6B1 IgG treatment) for scale.
**Figure 5.8. Mean NLP<sub>Pya</sub> production during *P. aphanidermatum* infection.** The nutrient solutions of hydroponically grown *C. annuum* plants were inoculated with *P. aphanidermatum* and the solutions were sampled daily over eight days. NLP<sub>Pya</sub> in the samples was quantified using the rabbit serum anti-NLP<sub>Pya</sub> assay. The solution from three separate *C. annuum* plants was sampled throughout the course of the experiment and triplicate samples were assayed; uninoculated growth medium from Day 8 was assayed to measure matrix effect. Bars above represent matrix-adjusted means of data compiled daily ± SEM.
5.3.5. Quantification of NLP$_{pya}$ in *P. aphanidermatum*-infected solutions

Using the CI-ELISA assay developed in Chapter 3, *P. aphanidermatum*-infected plant growth medium from three different hydroponic *C. annuum* plants was analysed for NLP$_{pya}$ content and quantity. Plant growth medium from uninoculated plants was also assayed to provide a measure of the matrix effect produced by plant nutrient solution. Standard curves were generated using known NLP$_{pya}$ concentrations and a log(inhibitor) vs. response model (Y=Bottom + [Top-Bottom]/[1+10^[X-LogIC50]]) in the program GraphPad Prism for Mac 5.00. Unknown NLP$_{pya}$ concentrations were interpolated from standard curves using their OD$_{450}$ readings from the assay; these values are presented in Figure 5.8.

Mean NLP$_{pya}$ content of the hydroponic nutrient solution on Day 0, the day the plants were inoculated with *P. aphanidermatum*, was calculated to be 40 nM. This was comparable with the uninoculated nutrient solution, which had a NLP$_{pya}$ content assessed to be 37 nM due to the aforementioned matrix effect. Thus, all calculated NLP$_{pya}$ concentrations were matrix-adjusted by subtracting the 37 nM attributed to this effect. By Day 1 the mean NLP$_{pya}$ content had increased from 3 to 11 nM. Over the next seven days, NLP$_{pya}$ levels increased (see Figure 5.8). By Day 8, assessed NLP$_{pya}$ content was 60 nM.

5.4. Discussion

To study the effect of NLP$_{pya}$ on plant growth and development, *N. tabacum* seeds were germinated on several different concentrations of NLP$_{pya}$. Decreased germination was observed from seeds planted on 5.0 µM NLP$_{pya}$, at a rate of 56%; this difference was
found to be statistically significant when compared to germination on MS∅, which was 89%. Lower concentrations of NLP$_{pya}$ did not have this effect on seed germination, when compared to germination rates on MS∅ (83% and 89% for 0.2 µM and 1.0 µM NLP$_{pya}$, respectively). Thus, it can be said that high concentrations of NLP$_{pya}$ (> 1.0 µM) affect the viability of *N. tabacum* seeds. Meir et al. (2009) observed a similar effect on *N. tabacum* seed germination with NLP$_{fo}$, albeit at a much higher concentration (285.7 µM). A marked dose-response effect of NLP$_{pya}$ was observed on the growth and development of *N. tabacum* seedlings that did germinate: seedling root lengths and dry weights were found to decrease proportionally as NLP$_{pya}$ concentration increased. ANOVA analysis of root length and dry weight data confirmed that the variation observed between MS∅ and NLP$_{pya}$ treatments was statistically significant. Hence, NLP$_{pya}$ supplemented growth medium inhibits the germination, growth and development of plant root and leaf structures. The most marked symptom of this effect was the abnormal root development observed on all concentrations of NLP$_{pya}$. Secondary or tertiary root structures and root hairs were not formed by any of the seedlings grown on NLP$_{pya}$; rather, seedlings developed a single elongated primary root. The stunted shoot development observed in seedlings grown on NLP$_{pya}$ as determined by their reduced dry weights was likely a side effect of this symptom, as these malformed root structures had a reduced ability to absorb nutrients. It is notable though, that while NLP$_{pya}$ had a profound effect on root development, no actual root browning or discolouration was observed during the 15-day course of these experiments. This may have been due the growth room temperature of 20°C, which is within the optimal temperature range for prevention of root browning (Sutton et al., 2006; Liu et al., 2007).
Though no dose-response effect was observed, infiltration of NLP\textsubscript{Pya} in \textit{N. benthamiana} leaf tissue also had a pronounced effect on plant health; all tested concentrations of NLP\textsubscript{Pya} (0.25 \(\mu\)M to 4.0 \(\mu\)M) produced the same level of necrosis from a 10 \(\mu\)L injection. Based on this observation, it can be concluded that NLP\textsubscript{Pya} is a very potent elicitor of necrosis in \textit{N. benthamiana} leaf cells. By contrast, a dose-response effect was observed with infiltrations of NLP\textsubscript{Bha} and NLP\textsubscript{Fo}. Concentrations of 0.25 \(\mu\)M and 0.5 \(\mu\)M produced a mottled effect of healthy-looking green tissue interspersed with yellowed or white necrotic-looking tissue. A similar effect was produced by Schouten et al. (2008) when they infiltrated \textit{N. benthamiana} leaves with low concentrations of NLP\textsubscript{Bc1} and NLP\textsubscript{Bc2} from \textit{B. cineria} (0.04 \(\mu\)M and 0.4 \(\mu\)M, respectively). As concentrations of NLP\textsubscript{Bha} and NLP\textsubscript{Fo} increased to 1.0 \(\mu\)M, the mottled effect gave way to more homogenous spots of necrotic tissue; this was also observed by Schouten et al. (2008) with NLP\textsubscript{Bc1} and NLP\textsubscript{Bc2} (with concentrations \(\geq\) 0.12 \(\mu\)M and \(\geq\) 1.2 \(\mu\)M, respectively). Based on these results, it can be concluded that NLP\textsubscript{Bha} and NLP\textsubscript{Fo} are powerful elicitors of necrosis at concentrations \(\geq\) 1.0 \(\mu\)M, but produce more variable necrosis at lower concentrations, i.e. at or below 0.5 \(\mu\)M. Thus of the NLP investigated, NLP\textsubscript{Pya} appears to be the most potent elicitor in this regard, in that it produces predictable necroses even at low concentrations.

Infiltration with NLP\textsubscript{Sscl} produced no observable necrosis even at concentrations of 8.1 \(\mu\)M (results not shown). The only cell damage observed in infiltrations with NLP\textsubscript{Sscl} was wounding at the injection site, which can be attributed to mechanical damage from the syringe and not necrotic damage from NLP\textsubscript{Sscl}. This result was verified by injections with PBS only, some of which also resulted in mechanical wounding of \textit{N. benthamiana}.
*benthamiana* leaves. Thus, despite possessing the NLP-heptapeptide motif and having structural homology to NLP<sub>Bha</sub>, NLP<sub>Fo</sub> and NLP<sub>Pya</sub>, NLP<sub>Sscl</sub> does not appear to be an elicitor of necrosis. NLP<sub>Sscl</sub> is also distinct from two other *S. sclerotiorum* NLPs, i.e. SSNep1 and SSNep2, both of which are expressed by *S. sclerotiorum* during infection and have been observed to cause necrosis in *N. benthamiana* leaves (Bashi et al., 2010). This key difference is likely due to NLP<sub>Sscl</sub> being a hypothetical protein; NLP<sub>Sscl</sub> was translated from a coding sequence identified in the *S. sclerotiorum* genome by its homology to the *F. oxysporum* NLP (NLP<sub>Fo</sub>) coding sequence. Expression of NLP<sub>Sscl</sub> during the course of *S. sclerotiorum* infection has not been detected. As such, NLP<sub>Sscl</sub> more closely resembles the unexpressed NLP genes encoded in *P. sojae*. In this species, possession of multiple NLP genes and pseudogenes has been observed; actual NLP genes may not necessarily be expressed during infection, and expressed NLPs in possession of all key residues can still lack necrotic ability (Dong et al., 2012). As the NLP<sub>Sscl</sub> coding sequence is the correct size, lacks introns, and shows homology to other established NLPs genes, it is unlikely that it is a pseudogene. Rather, based on its correct size and structure, and retention of the key GHRHDWE motif, it is most likely an actual NLP gene that is no longer expressed by *S. sclerotiorum* during infection.

Based on its high affinity and cross-reactivity to NLPs (established in Chapter 3), the ability of anti-NLP<sub>Pya</sub> rabbit serum to neutralize NLP-induced necrosis was explored. By pre-incubating NLP<sub>Bha</sub> and NLP<sub>Pya</sub> with anti-NLP<sub>Pya</sub> rabbit serum, it was found that the serum was able to eliminate the necroses produced by these proteins. Pre-incubating NLP<sub>Bha</sub> or NLP<sub>Pya</sub> with pre-immune rabbit serum had no effect on necrosis, indicating that neutralization was indeed due to the anti-NLP<sub>Pya</sub> antibodies in the hyperimmune
rabbit serum. By contrast, the necroses produced by NLP<sub>Fo</sub> were not neutralized by anti-NLP<sub>Py</sub> rabbit serum. This was consistent with the reduced ability of the serum to bind NLP<sub>Fo</sub> (discussed in Chapter 3). The ability of the serum to neutralize NLP<sub>Bha</sub>- and NLP<sub>Py</sub>-induced necrosis, however, was limited: serum diluted ¼ or ½ in PBS was unable to mitigate the necrotic effects of NLP<sub>Bha</sub> and NLP<sub>Py</sub>, and higher concentrations of these NLPs (4.0 µM) could not be neutralized with undiluted serum (results not shown). Pre-incubation was also necessary for neutralization, as NLP<sub>Bha</sub> and NLP<sub>Py</sub> retained their ability to cause necrosis when co-infiltrated with anti-NLP<sub>Py</sub> serum immediately after mixing; the necroses produced from these infiltrations had the mottled appearance associated with partial necrosis (results not shown).

6B1 IgG was also assessed for its ability to neutralize NLP-induced necrosis. Co-infiltrating NLP<sub>Bha</sub> or NLP<sub>Py</sub> with 6B1 IgG at a low concentration (0.25 µM) could reduce but not eliminate necrosis, as indicated by the mottled appearance of the plant tissue. Co-infiltrating NLP<sub>Bha</sub> or NLP<sub>Py</sub> with 6B1 IgG at a higher concentration (0.5 µM) resulted in complete elimination of necrosis. This effect was also observed with NLP<sub>Fo</sub>, albeit at a reduced capacity. Co-infiltration with 6B1 IgG at 0.25 µM showed no effect on necrosis, but co-infiltration with 6B1 IgG at 0.5 µM resulted in a partial reduction of necrosis, as indicated by the mottled appearance of the infiltrated plant tissue. This was unexpected, given that the apparent binding affinity of 6B1 IgG to NLP<sub>Fo</sub> was actually lower than that of anti-NLP<sub>Py</sub> rabbit serum (based on their IC50s). This discrepancy may be explained by differences in the quantity of anti-NLP antibody being administered in the co-infiltrations. The actual quantity of antibody in anti-NLP<sub>Py</sub> rabbit serum capable of binding NLP<sub>Py</sub> was technically unknown, due to the polyclonal nature of the serum.
Furthermore, not all anti-NLP<sub>Pya</sub> antibodies would be anti-NLP antibodies (as discussed in Chapter 3). This contrasted with 6B1 IgG, which was an anti-NLP monoclonal antibody produced <i>in planta</i> and quantified accurately. Thus, it is possible that 6B1 IgG was administered at a concentration higher than the anti-NLP antibodies present in anti-NLP<sub>Pya</sub> serum, thereby compensating for the reduced binding capacity of 6B1 IgG (relative to the serum). The ability of 6B1 IgG to neutralize NLP-induced necrosis corroborated the observation by Ottmann et al. (2009) that the GHRHDWE motif is necessary for NLP activity. It also demonstrated that NLP activity could be sufficiently neutralized by an antibody binding only this region.

Using the anti-NLP rabbit serum assay developed in Chapter 3, the NLP<sub>Pya</sub> levels in <i>P. aphanidermatum</i>-infected hydroponic plant growth medium were assessed. A matrix effect due to the plant nutrient medium was observed, since the matrix created a false positive value of 37 nM for uninoculated nutrient solution and 40 nM for nutrient solutions immediately following inoculation with <i>P. aphanidermatum</i>. Thus, all values calculated were adjusted to account for the matrix effect.

When grown at temperatures of 24-32°C, root browning in <i>P. aphanidermatum</i>-infected plants begins by Day 3 (Liu et al., 2007); this was consistent with the <i>C. annuum</i> plants studied in this experiment (results not shown). As the appearance of root browning in infected plants coincides with the <i>Pythium</i> transition from biotrophy to necrotrophy (Sutton et al., 2006), and the transition from biotrophy to necrotrophy coincides with NLP production (Qutob et al., 2002), it would follow that NLP<sub>Pya</sub> production coincides with root browning. The assayed levels of NLP<sub>Pya</sub> showed just that, reaching detectable levels on Day 3 of the experiment. NLP<sub>Pya</sub> levels on Day 3 increased to 20 nM, just
below the anti-NLP$_{pya}$ rabbit serum LoD of 22 nM. On Days 4 and 5, the NLP$_{pya}$ levels were calculated to be 27 and 23 nM (respectively). As it is unlikely that NLP$_{pya}$ levels actually dropped between Day 4 and Day 5, these measurements can be explained by a lack of precision due to the NLP$_{pya}$ levels being so close to the LoD of the assay. However, the measured NLP$_{pya}$ levels for Days 3-5 were above the LoD indicating that when \textit{P. aphanidermatum} transitions to necrotrophy and root browning begins, the concentrations are approximately 25 nM. This was nearly ten times lower than the lowest concentration studied in the NLP$_{pya}$ dose-response experiments discussed earlier. In these experiments, it was observed that 200 nM NLP$_{pya}$ had a considerable effect on root development, but produced no root browning. Between Days 5 and 8, the concentration of NLP$_{pya}$ increased by approximately 10 nM per day, reaching 60 nM by the end of the experiment. As this was well above the LoD of anti-NLP$_{pya}$ rabbit serum, these measurements were more precise than those from Day 3. Root browning in \textit{P. aphanidermatum}-infected plants typically plateaus between Days 7 and 9 (Liu et al., 2007). No visible increase in root browning was observed after Day 7 of the experiment (results not shown), but NLP$_{pya}$ levels were still increasing steadily when the experiment was terminated. This suggests that the NLP$_{pya}$ concentration was not directly correlated with the degree of root browning. However, due to the limited time period of the experiment, it is difficult to draw any firm conclusions about this relationship. The dose-response data indicate that NLP$_{pya}$ causes root malformation by itself, but no actual root browning without the presence of the pathogen \textit{P. aphanidermatum}. Since NLP$_{pya}$ is known to induce accumulation of 4-hydroxybenzoic acids in plant cells (Koch et al., 1998), it is possible that \textit{P. aphanidermatum} either releases or stimulates the release of
other compounds necessary for the formation of the specific phenolics responsible for the root browning symptom in plants. Further experimentation is required to determine if NLP\textsubscript{Pya} concentrations in the later stages of \textit{P. aphanidermatum} infection coincide with any plant disease symptoms.

Several aspects of NLPs and anti-NLP antibodies were studied in this chapter, and from these experiments several conclusions can be drawn. Even without their respective pathogens, NLPs can have pronounced adverse effects on plants by causing necroses in leaf tissues and, in the case of NLP\textsubscript{Pya}, by inhibiting plant development as a whole. This was true of all the NLPs studied, with the exception of the hypothetical NLP translated from the \textit{S. sclerotiorum} genome. It was determined that the anti-NLP\textsubscript{Pya} rabbit serum examined in Chapter 3 was capable of neutralizing necroses elicited by NLP\textsubscript{Bha} and NLP\textsubscript{Pya} but not NLP\textsubscript{Fo}. The IgG 6B1, which was engineered to bind all NLPs, was capable of neutralizing all three. Finally, it was determined that the assay developed in Chapter 3 is capable of diagnosing early-stage \textit{P. aphanidermatum} infection in hydroponic plant systems. Due to the assay’s limit of detection, however, NLP\textsubscript{Pya} levels cannot be accurately quantified until the later stages of infection.
6. **Overall Conclusions and Future Research Directions**

The antibodies and assays developed and discussed in this thesis can be regarded in two different contexts: as proof-of-concept models, and as diagnostic and remedial tools. The concepts explored in this thesis were antibody cross-reactivity based on a conserved structural motif, differences in antibody affinity between antigens with a conserved motif, and neutralization of antigen activity. These concepts were either proven or demonstrated throughout the preceding chapters, as discussed below. The diagnostic applications evaluated in this thesis were cross-reactive polyclonal and monoclonal assays for detection of multiple NLP antigens, and a polyclonal assay for quantification of these NLPs. The remedial applications evaluated were the capabilities of these cross-reactive polyclonal and monoclonal antibodies to neutralize these plant pathogenic elicitors and their associated necroses. These abilities were demonstrated with varying degrees of success and practicality, as examined in this chapter.

Chapter 3 of this thesis established that antiserum raised against one specific NLP could be used to bind and detect other NLPs in a quantifiable fashion. Furthermore, it was shown that the cross-reactivity of this serum to the four NLPs was due to its binding to the conserved NLP-heptapeptide motif present in all known NLPs. The result was a polyclonal assay that detected, at nM concentrations, all four NLPs tested. This assay can be considered a success as a proof-of-concept model: the quantifiable cross-reactivity of the assay served to prove that polyclonal serum raised against one NLP could bind multiple NLP species through their shared structural motif. In terms of an effective diagnostic tool, the polyclonal assay was again a success in that the polyclonal serum was
capable of binding, at nM concentrations, and detecting all NLPs tested, as indicated by the limits of detection and IC50s of the serum with respect to each NLP. As demonstrated in Chapter 5, the polyclonal assay was also capable of detecting and quantifying NLP_{Pya} in *P. aphanidermatum*-infected hydroponic nutrient solutions. Based on the LoD of the assay, NLP_{Pya} could be accurately quantified by Day 3 of infection. Thus, the polyclonal assay could potentially diagnose *P. aphanidermatum* infection as early as Day 3, coinciding with the start of root browning symptoms. However, the polyclonal assay falls short as a practical diagnostic tool due to the finite supply of the antiserum. Furthermore, the assay cannot be altered to improve affinity (e.g. via antibody affinity maturation) and/or decrease the LoD for detection of the NLP prior to three days post infection.

Chapter 4 further investigated the concept of cross reactivity by raising monoclonal antibodies against one conserved antigenic target, namely the NLP-heptapeptide. The resulting mAb was studied in several different antibody formats and in each format was found to have nM to µM affinity for the four NLPs tested. Thus, the monoclonal anti-NLP assay was also a success as a proof-of-concept. As a diagnostic tool, the monoclonal IgG assay was a moderate success. The IC50 of 6B1 IgG with respect to NLP_{Bha}, NLP_{Pya} and NLP_{Ssc1} demonstrated that the assay was capable of binding and detecting some NLPs in nM quantities. However, the high IC50 of 6B1 IgG with respect to NLP_{Fo} demonstrated that the assay did not bind all NLPs with the same relative affinity. As such, the monoclonal assay may not be able to detect all NLPs at concentrations in the 1 to 100 nM range.

Chapter 5 evaluated the abilities of anti-NLP antibodies to neutralize NLP-induced necroses in plant tissues. Both the polyclonal serum and the monoclonal IgG
were successful in this respect, albeit to different degrees. The polyclonal serum was capable of completely neutralizing the necroses produced by NLP$_{\text{Bha}}$ and NLP$_{\text{Pya}}$, but only when pre-incubated, undiluted, with the NLPs. Additionally, it had no effect on NLP$_{\text{Fo}}$-induced necrosis. The anti-NLP IgG was more successful in this regard, in that at a low concentration ($0.5 \, \mu\text{M}$) it was capable of completely neutralizing NLP$_{\text{Bha}}$ and NLP$_{\text{Pya}}$, and was able to partially neutralize NLP$_{\text{Fo}}$. As a proof-of-concept, it was demonstrated with the polyclonal serum that anti-NLP antibodies could prevent NLP-induced necrosis. Using the IgG this concept was taken a step further when it was demonstrated that NLP activity could be sufficiently neutralized by an antibody binding specifically to the NLP-heptapeptide region.

In terms of a remedial tool, 6B1 mAb IgG is more practical than the polyclonal serum. A mAb IgG is sustainable, in that it can be reliably produced \textit{in vitro} and modified by any number of affinity maturation or recombinant DNA techniques. However, 6B1 mAb IgG is limited by its low affinity to the NLPs (relative to its scFv precursor and the polyclonal serum). For instance, given the low concentrations of NLP$_{\text{Pya}}$ present in \textit{P. aphanidermatum}-infected hydroponic solutions, it is doubtful that 6B1 mAb IgG would be capable of detecting early-stage infection in a greenhouse system. An affinity maturation step would likely be necessary for 6B1 mAb IgG to be a reliable diagnostic tool. Additionally, the vast quantities of 6B1 mAb IgG that would be required to neutralize NLP$_{\text{Pya}}$ in a hydroponic greenhouse system preclude production of exogenous 6B1 from being a viable option.

The ability of an anti-NLP mAb (i.e. 6B1 IgG) to mitigate \textit{P. aphanidermatum} disease symptoms in plants has yet to be demonstrated. Since NLP$_{\text{Pya}}$ is a key
determinant in *P. aphanidermatum* pathogenesis, and the only NLP produced by this organism, 6B1 IgG has the potential to be a useful pesticide against this pathogen. 6B1 could be a similarly effective pesticide against other organisms relying on a single NLP for pathogenesis; for example, *Pectinobacterium carotovorum* subsp. *carotovorum*, provided that there is cross reactivity to NLP<sub>Pcc</sub> (Mattinen et al., 2004). This application may not extend to all NLP-producing pathogens, as many of these organisms produce non-NLP elicitors, and some NLPs do not play a role in an organisms’ pathogenesis (i.e. those that infect monocots) (Gijzen and Nürnberger, 2006). Also, many organisms produce multiple NLPs, such as species of *Phytophthora*, which could overwhelm the neutralization capabilities of a single anti-NLP antibody in a hydroponic system (Dong et al, 2012). It would be possible to determine this by individually evaluating the binding capacity of 6B1 IgG with respect to each NLP, as well as the levels of each NLP produced in hydroponic infection. The capability of 6B1 IgG to mitigate plant disease could be investigated as a proof-of-concept against susceptible pathogens like *P. aphanidermatum* or *P. carotovorum* subsp. *carotovorum*; this could be accomplished using a small-scale hydroponic system (such as the experiment described in Section 5.2.4) with the addition of exogenous antibody. These experiments, however, were beyond scope of this thesis.

While formidable, the practicality issues surrounding 6B1 mAb IgG expression are not insurmountable. Rather than produce exogenous antibody for a greenhouse environment, 6B1 IgG could be cloned for stable expression in susceptible plants. However, this strategy would require cloning the 6B1 IgG transgenes into each different species of susceptible plant. It would also require development of transgenic plant lines to
ensure reliable antibody expression in plant roots, where *P. aphanidermatum* colonization occurs. Alternatively, 6B1 could be produced within hydroponic nutrient medium via a bacterial expression system. The resilient nature and commensal ability of many *Pseudomonas* species would make them suitable for protein expression in a hydroponic environment. In particular, the antagonistic relationship between *Pseudomonas chlororaphis* and *P. aphanidermatum* makes this species an attractive candidate for this purpose (Liu et al., 2007). Additionally, the development of vectors that enable stable transformation of foreign genes into the genome of *Pseudomonas* spp. eliminates the need for continuous antibiotic selection (Choi and Schweizer, 2006). This would essentially create an antibody-armed strain of *Pseudomonas chlororaphis* needing only to be inoculated into a susceptible hydroponic system.

The monoclonal and polyclonal antibodies and antibody-based assays developed and investigated in this thesis were valuable models for the proofs of several concepts, as discussed above. These antibodies and assays were also demonstrated to be useful tools for the detection, quantification, and neutralization of the NLPs and their associated necroses in plants.
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APPENDIX

Appendix 1: NLP<sub>Pya</sub> production during <i>P. aphanidermatum</i> infection.

The nutrient solution of hydroponically grown <i>C. annuum</i> plants was inoculated with <i>P. aphanidermatum</i> and the solution was sampled daily over a course of eight days. Using the rabbit serum anti-NLP<sub>Pya</sub> assay, NLP<sub>Pya</sub> in the samples was quantified. The nutrient solution of three separate <i>C. annuum</i> plants was sampled throughout the course of the experiment and samples were assayed in triplicate. Uninoculated hydroponic growth medium was also assayed to measure absorbance due to plant growth medium (matrix effect). Means were matrix-adjusted (MA-Mean) by subtracting the matrix effect value for final concentrations. Concentrations are presented in µm.

<table>
<thead>
<tr>
<th></th>
<th>Plant I</th>
<th>Plant II</th>
<th>Plant III</th>
<th>Mean</th>
<th>MA-Mean</th>
</tr>
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<tbody>
<tr>
<td>Day 0</td>
<td>0.038</td>
<td>0.046</td>
<td>0.035</td>
<td>0.040</td>
<td>0.003</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.057</td>
<td>0.055</td>
<td>0.043</td>
<td>0.052</td>
<td>0.015</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.045</td>
<td>0.051</td>
<td>0.053</td>
<td>0.050</td>
<td>0.013</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.048</td>
<td>0.058</td>
<td>0.063</td>
<td>0.057</td>
<td>0.020</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.048</td>
<td>0.081</td>
<td>0.065</td>
<td>0.064</td>
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</tr>
<tr>
<td>Day 5</td>
<td>0.050</td>
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<td>0.068</td>
<td>0.060</td>
<td>0.023</td>
</tr>
<tr>
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<td>Day 7</td>
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<td>0.084</td>
<td>0.088</td>
<td>0.051</td>
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<td>0.101</td>
<td>0.089</td>
<td>0.097</td>
<td>0.060</td>
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<tr>
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<td>0.031</td>
<td>0.036</td>
<td>0.037</td>
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