SELECTION, CHARACTERIZATION, AND APPLICATION OF A MYCOTOXIN-SPECIFIC SINGLE DOMAIN ANTIBODY FRAGMENT.

A Thesis
Presented to
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of
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by
PATRICK JOHN DOYLE

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ABSTRACT

SELECTION, CHARACTERIZATION, AND APPLICATION OF A MYCOTOXIN-SPECIFIC SINGLE DOMAIN ANTIBODY FRAGMENT.

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University of Guelph, 2009

Advisor:  Professor J.C. Hall

15-Acetyl-deoxynivalenol (15-AcDON) is a low molecular weight (M.W. = 338 Da) sesquiterpenoid trichothecene mycotoxin associated with Fusarium Head Blight (FHB), a global disease of small grain cereals. Estimated economic losses from the most recent Canadian and US FHB epidemic during the mid 1990s range from $1.3 to $3.0 billion USD, depending on valuations assigned to reduced crop yield and mycotoxin contamination of food and feed. The accumulation of DON and related trichothecene mycotoxins within harvested grain is subject to stringent regulation as both toxins pose dietary health risks to humans and animals. A property of 15-AcDON, and structurally-similar trichothecene mycotoxins that are intimately involved in FHB pathogenesis, is the propensity to inhibit peptidyl transferase activity and thus inhibit protein synthesis within infected host-plant cells. This effect, coupled with secondary phytotoxic effects, act as mechanisms of virulence for pathogenic Fusarium species.

A single-domain variable heavy chain (V\textsubscript{H}H) recombinant antibody (rAb) fragment specific to 15-AcDON was isolated from a hyper-immunized phagemid library. The dominant clone (NAT-267) was expressed and purified as a V\textsubscript{H}H monomer with a competitive binding affinity of 1.24 µM. The gene encoding the NAT-267 camelid single-domain antibody fragment (V\textsubscript{H}H) was expressed within the methylotrophic yeast
*Pichia pastoris*. Mycotoxin-mediated cytotoxicity was assessed by continuous measurement of cellular growth over time. At equivalent doses, 15-AcDON was significantly more toxic to wild-type *P. pastoris* than was DON, which, in turn, was more toxic than 3-AcDON. Intracellular expression of this toxin-specific V_{H}H ‘intrabody’ within *P. pastoris* conveyed significant (p = 0.01) resistance to 15-AcDON cytotoxicity at doses ranging from 20 to 100 µg·mL\(^{-1}\), making this the first report of V_{H}H-based sequestration of a haptenic mycotoxin. Furthermore, a biochemical transformation of DON to 15-AcDON was documented, which explained significant attenuation of the efficacy of DON at 100 and 200 µg·mL\(^{-1}\). This “proof of concept” model suggests that *in planta* V_{H}H expression may lead to enhanced tolerance to mycotoxins and thereby serve as a novel tool to help limit *Fusarium* infection of commercial agricultural crops such as wheat and barley that are engineered to express this mycotoxin-specific V_{H}H.
PREFACE

This dissertation contains three manuscripts prepared for publication within a peer-reviewed text book and two scientific journals. Each manuscript is listed below with the respective publication status.

CHAPTER TWO

CHAPTER FOUR

CHAPTER FIVE
ACKNOWLEDGEMENTS

I would like to thank my graduate committee for their trust, wisdom, and patience in helping to guide me through this degree. Chris, Marc, Roger, and Steve... it has been a privilege to learn from you and work in your labs. Thank-you for your friendship and help along the journey! Also, in alphabetical order... special regards and sincere thanks to: Anne Hermans, Barb Blackwell, Charles Seguin, Christian Luebbert, Claudia Sheedy, Garth Gourlay, Ginette Dubuc, Gord Furzer, Greg Hussack, Hanaa Saeed, Hong Tong-Sevinc, Jamshid Tanha, Joy Roberts, Karen Gourlay, Keith Solomon, Larry Erickson, Linda Veldhuis, Mehdi Arbabi-Ghahroudi, Mike McLean, Nathalie Gaudette, Nina Weisser, “Osama-Bin Llama”, Shannon Ryan, Tomoko Hirama, and Yonghong Guan.

Sincere gratitude and thanks to my friends at Syngenta Crop Protection Canada Inc. for the tremendous support and encouragement. Special thanks to Marian Stypa. You believed in me and were always there for me (and my family) right from the start! To Norm Dreger, thanks for adopting me into the “Business Development” family and for the huge... patience, support, and of course for the valuable insights that you have taught me about people. I would also like to thank Ms. Laurie Vescio and Mr. Jay Bradshaw for the great support and words of kind encouragement over these six + years.

Finally, to my family. Thank-you so much... Nadine, you held our family together all those times that I was away. Even today, I never worry about the most important stuff because I always know that you have it covered. Mom and Dad... thanks for all the advice and insights provided during all those late-night phone calls to Calgary. Finally, to my kids... Liam and Danielle, I have watched you grow alongside this PhD project. I am very sad that doing this took a lot of my time away from you. Danielle and Liam, you always were, and will always be in my heart. All those times that I was working on”school stuff”, you were always the light and my joy that kept me going forward. I love you both with all my heart...!
Left to Right: “Osama-Bin” Llama; Danielle (hiding), Liam, and Nadine
NOTE ABOUT WHY

In the preface of: Fusarium Head Blight of Wheat and Barley, the editors (Kurt J. Leonard and William R. Bushnell) make the following dedication.

“This book is dedicated to those scientists who labor to solve the Fusarium head blight problem, despite knowing full well that easier paths may be taken in the pursuit of peer recognition and scientific acclaim.”

I do not believe that this dedication was a challenge (or ‘snub’) to the challenges, relevance, or prestige of the vast number of other disciplines and technical pursuits within modern science. To me, Drs. Leonard and Bushnell were simply trying to acknowledge what “bad actors” FHB and its toxin problem(s): i.e., DON... + acetylated “brother’s”, 15-AcDON and 3AcDON can be.

For the record, my interest in FHB goes back to the epidemic in 1993/1994/1995 (esp. 1994!) when I ran a small research farm in southern Manitoba. FHB may be a cyclical disease that comes along only once in a long while, but when it gets going, it is a huge problem that affects many people, especially farmers. Like Leonard and Bushnell, I take my hat off to, and admire, those scientists who contribute to this field.
LIST OF ABBREVIATIONS

15-AcDON  15-Acetyl-Deoxynivalenol
15-DON-BSA  Bovine Serum Albumin Conjugated to C-15 of DON
15-DON-HRP  Horse Radish Peroxidase Conjugated to C-15 of DON
15-DON-OVA  Ovalbumin Conjugated to C-15 of DON
3-AcDON  3-Acetyl-Deoxynivalenol
AAFC  Agriculture and AgriFood Canada
Ab  Antibody
Ag  Antigen
AOX  Alcohol Oxidase Promoter
AP  Alkaline Phosphatase
ARM  Antibody-Ribosome-mRNA Complex
BCIP  5-Bromo-4-Chloro-3-Indolyl Phosphate
BSA  Bovine Serum Albumin
CD-ELISA  Competitive Direct ELISA
cDNA  Complementary DNA
CDR  Complementarity Determining Region
C\textsubscript{H}  Constant Domain of the Heavy Chain
cIgG  Conventional Immunoglobulin Gamma (γ) Isotype Abs
C\textsubscript{L}  Constant Domain of the Light Chain
Da  Dalton
DAI  Days After Inoculation
DAPI  4’,6-diamidino-2-phenylindole dihydro-chloride
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<td>DAS</td>
<td>Diacetoxyscirpenol</td>
</tr>
<tr>
<td>DMAP</td>
<td>Dimethylaminopyridine</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DON</td>
<td>Deoxynivalenol</td>
</tr>
<tr>
<td>dsFv</td>
<td>Disulfide-Stabilized Fv</td>
</tr>
<tr>
<td>ECORC</td>
<td>Eastern Cereal and Oilseed Research Center</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment, Antigen Binding Region of an Antibody</td>
</tr>
<tr>
<td>FDK</td>
<td><em>Fusarium</em> Damaged Kernels</td>
</tr>
<tr>
<td>Ff</td>
<td>Filamentous Bacteriophage</td>
</tr>
<tr>
<td>FHB</td>
<td><em>Fusarium</em> Head Blight</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescence Polarization</td>
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<tr>
<td>FPA</td>
<td>Fluorescence Polarization Assay</td>
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<td>FR</td>
<td>Framework Region</td>
</tr>
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<td>Fv</td>
<td>Variable Fragment of an Antibody</td>
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<td>GC/MS</td>
<td>Gas Chromatogram / Mass Spectrometry</td>
</tr>
<tr>
<td>HCAb</td>
<td>Heavy Chain Antibody</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HPI</td>
<td>Hours Post Infection</td>
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<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half Maximal Inhibitory Concentration</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin Alpha (α) Isotype Antibody</td>
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<td>IgM</td>
<td>Immunoglobulin Mu (μ) Isotype Antibody</td>
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<tr>
<td>IMAC</td>
<td>Immobilized Metal Affinity Chromatography</td>
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<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
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<td>JECFA</td>
<td>Joint Expert Committee on Food Additives</td>
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<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>Equilibrium Dissociation Constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median Lethal Dose</td>
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<tr>
<td>LSD</td>
<td>Least Significant Difference</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
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<td>MCS</td>
<td>Multiple Cloning Site</td>
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<td>MGYH</td>
<td>Minimum Glycerol Medium with Histidine</td>
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<td>MMH</td>
<td>Minimal Methanol Medium</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MW</td>
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<tr>
<td>NBT</td>
<td>Nitro Blue Tetrazolium Chloride</td>
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<tr>
<td>NEO</td>
<td>Neosolaniol</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<td>NRCC</td>
<td>National Research Council of Canada</td>
</tr>
<tr>
<td>NSERC</td>
<td>National Sciences and Engineering Research Council</td>
</tr>
<tr>
<td>OAc</td>
<td>Acetyl Ester (-OCH₃)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>-------------</td>
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<tr>
<td>OD&lt;sub&gt;620&lt;/sub&gt;</td>
<td>Optical Density at 620nm</td>
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<td>OIsoval</td>
<td>Isovalerate Ester (-OCOCH&lt;sub&gt;2&lt;/sub&gt;CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;)</td>
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<td>OMAFRA</td>
<td>Ontario Ministry of Agriculture, Food and Rural Affairs</td>
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<td>OVA</td>
<td>Ovalbumin</td>
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<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>PBL</td>
<td>Peripheral Blood Lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline Tween</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>pIII</td>
<td>Minor Coat Protein of Filamentous Phage</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl Fluoride</td>
</tr>
<tr>
<td>PMTDI</td>
<td>Provisional Maximum Tolerable Daily Intake</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
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<tr>
<td>pVIII</td>
<td>Major Coat Protein of Filamentous Phage</td>
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<td>rAb</td>
<td>Recombinant Antibody</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<td>RPL3</td>
<td>Ribosomal Protein L3</td>
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<td>SAR</td>
<td>Systemic Acquired Resistance</td>
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<td>scFv</td>
<td>Single Chain Fv</td>
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<td>sdAb</td>
<td>Single Domain Ab</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
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<td>ssDNA</td>
<td>Single-Stranded DNA</td>
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<td>StEP</td>
<td>Staggered Extension Process</td>
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<td>TBST</td>
<td>Tween Buffered Saline</td>
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<tr>
<td>TEA</td>
<td>Triethylamine</td>
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<td>TFAA</td>
<td>Trifluoroacetic anhydride</td>
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<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>UGT</td>
<td>UDP Glycosyltransferase</td>
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<tr>
<td>USD</td>
<td>United States Dollars</td>
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<tr>
<td>$V_{H}$</td>
<td>Variable Domain of the Heavy Chain</td>
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<td>$V_{H\ H}$</td>
<td>Variable Region on Single-Domain Heavy Chain Ab</td>
</tr>
<tr>
<td>$V_{L}$</td>
<td>Variable Domain of the Light Chain</td>
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<td>YPD</td>
<td>Yeast Potato Dextrose media</td>
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<td>YPDS</td>
<td>Yeast Extract Peptone Dextrose Sorbitol Media</td>
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<tr>
<td>ΔAUC</td>
<td>Differential Area Under Curve</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td>PREFACE</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>PICTURE</td>
<td>iii</td>
</tr>
<tr>
<td>NOTE ABOUT WHY</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xviii</td>
</tr>
<tr>
<td>1 INTRODUCTION, OBJECTIVES, AND HYPOTHESIS</td>
<td>1</td>
</tr>
<tr>
<td>2 LITERATURE REVIEW 1: FUSARIUM HEAD BLIGHT AND TRICHOTHECENE MYCOTOXINS: NOVEL TARGETS FOR RECOMBINANT ANTIBODY TECHNOLOGY</td>
<td>6</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>6</td>
</tr>
<tr>
<td>2.2 Fusarium Head Blight</td>
<td>8</td>
</tr>
<tr>
<td>2.2.1 Disease Cycle</td>
<td>8</td>
</tr>
<tr>
<td>2.2.1.1 Inoculum</td>
<td>10</td>
</tr>
<tr>
<td>2.2.1.2 Environment</td>
<td>11</td>
</tr>
<tr>
<td>2.2.1.3 Host Crop</td>
<td>11</td>
</tr>
<tr>
<td>2.2.2 Pathways of FHB Pathogenesis</td>
<td>13</td>
</tr>
<tr>
<td>2.2.2.1 Fungal Colonization</td>
<td>13</td>
</tr>
<tr>
<td>2.2.2.2 Systemic Infection</td>
<td>15</td>
</tr>
</tbody>
</table>
2.2.3  FHB Control Strategies .................................................................16
   2.2.3.1  Agronomic and Cultural Practices ............................................16
   2.2.3.2  Fungicides ..............................................................................17
   2.2.3.3  Biological Control Agents .........................................................17
   2.2.3.4  Crop Breeding .........................................................................18
2.2.4  Classifications of FHB Resistance ..................................................19
2.3  Trichothecene Mycotoxins ..................................................................20
   2.3.1  Mode of Action ............................................................................21
   2.3.2  Mechanisms of Fungal Protection ..................................................22
   2.3.3  Effects on Plants ..........................................................................23
2.4  DON, 3-AcDON, and 15-AcDON .........................................................24
   2.4.1  Contamination of Food and Feed ...................................................25
   2.4.2  Mechanism of Virulence for FHB ...................................................26
2.5  Transgenic Approaches to Limit Trichothecene Toxicity .......................28
   2.5.1  Inhibition of Trichothecene Biosynthesis ........................................29
   2.5.2  Alteration of Target Protein ..........................................................30
   2.5.3  Metabolic Trichothecene Detoxification ..........................................31
   2.5.4  Reduction of Intracellular Concentrations .......................................33
   2.5.5  Plantibodies and Mimotopes ..........................................................33
3  LITERATURE REVIEW 2: ANTIBODY ENGINEERING IN
AGRICULTURAL BIOTECHNOLOGY ..........................................................36
   3.1  Introduction .....................................................................................36
   3.2  Recombinant Antibody Fragments .....................................................37
3.2.1 Fab Fragment ................................................................. 38
3.2.2 scFv Fragment ............................................................. 39
3.2.3 \( V_{H}H \) Fragment ......................................................... 42

3.3 Development and Types of Antibody Libraries ................................. 44
3.3.1 Immunized Libraries ....................................................... 44
3.3.2 Naïve Libraries ............................................................. 45
3.3.3 Synthetic / Semi-Synthetic Libraries ................................ 46

3.4 Display Systems .................................................................. 47
3.4.1 Phage Display ................................................................. 47
3.4.1.1 Filamentous Bacteriophage ........................................ 48
3.4.1.2 Phage vs. Phagemid Vectors ....................................... 49
3.4.1.3 Panning .................................................................. 52
3.4.2 Bacteria Display .............................................................. 54
3.4.3 Yeast Display .................................................................. 54
3.4.4 Ribosome Display ........................................................... 55

3.5 Optimization of Recombinant Antibodies ....................................... 57
3.5.1 Optimized Panning Procedures .......................................... 57
3.5.2 \textit{In vitro} Affinity Maturation ......................................... 58
3.5.2.1 Random Mutagenesis .................................................. 59
3.5.2.1.1 Error-Prone PCR ................................................... 59
3.5.2.1.2 Bacterial Mutator Strains ....................................... 59
3.5.2.2 Focused Mutagenesis ................................................... 60
3.5.2.2.1 Site-Directed Mutagenesis ..................................... 60
4.3.1  Reagents and Solutions .................................................................86
4.3.2  Immunogen and Llama Immunization ..........................................87
4.3.3  Fractionation of Polyclonal Serum .............................................88
4.3.4  Confirmation of Titer ..................................................................89
4.3.5  Library Construction ..................................................................90
4.3.6  V_HH Library Panning ............................................................92
4.3.7  Phage ELISA ...........................................................................93
4.3.8  Colony PCR and Sequencing .....................................................94
4.3.9  Monomer Expression and Purification ..........................................94
4.3.10 Pentamerization of V_HH Monomer ..........................................95
4.3.11 Pentamer Expression and Purification ........................................96
4.3.12 Determination of Binding Affinity .............................................96
    4.3.12.1 Fluorescence Polarization Assay ......................................96
    4.3.12.2 Surface Plasmon Resonance ...........................................98
    4.3.12.3 Competitive Inhibition ELISA ........................................99
4.4  Results .......................................................................................100
    4.4.1 Polyclonal Response ...............................................................100
        4.4.1.1 Direct ELISA ................................................................100
        4.4.1.2 Fluorescence Polarization ..........................................101
    4.4.2 HCAb and cIgG Response ....................................................102
        4.4.2.1 Protein A and G Chromatography ................................102
        4.4.2.2 ELISA on Polyclonal Serum Fractions .......................102
    4.4.3 Library Construction .............................................................103
4.4.4 Panning and Phage ELISA .................................................................103
4.4.5 V_{H}H Sequence Results .................................................................104
4.4.6 Expression of Soluble V_{H}H Protein ...............................................105
4.4.7 V_{H}H Affinity Measurements ..........................................................105
  4.4.7.1 Fluorescence Polarization ..........................................................105
  4.4.7.2 Surface Plasmon Resonance .......................................................106
  4.4.7.3 CI ELISA .................................................................................110
4.5 Discussion .........................................................................................110
4.6 Conclusions ......................................................................................118
4.7 Acknowledgements ............................................................................119

5 RESEARCH CHAPTER 2: INTRACELLULAR EXPRESSION OF A
SINGLE-DOMAIN ANTIBODY REDUCES CYTOTOXICITY OF 15-
ACETYL-DEOXYNIVALENOL IN YEAST .........................................................120

  5.1 Abstract ..........................................................................................120
  5.2 Introduction .....................................................................................121
  5.3 Experimental Procedures .................................................................124
    5.3.1 V_{H}H Genes .............................................................................124
    5.3.2 Transformation ..........................................................................125
    5.3.3 Induction of V_{H}H Expression ..................................................126
    5.3.4 Preparation of Soluble V_{H}H Extracts and Western Blot Analysis ....126
    5.3.5 Cytotoxicity Assays .....................................................................127
    5.3.6 Data Analysis ..............................................................................128
    5.3.7 Quantitative Western Blot Analysis ..........................................129
    5.3.8 V_{H}H Immunolocalization .......................................................131
5.3.9 Mycotoxin Biotransformation Assays ............................................. 132

5.4 Results ........................................................................................................... 134

5.4.1 Sensitivity to Ribotoxin Treatments ......................................................... 134

5.4.2 Transformation and V_{H}H Intrabody Expression ................................. 135

5.4.3 Quantitative Western Blot Assay ............................................................. 135

5.4.4 Cytotoxicity Assays .................................................................................. 137

5.4.4.1 15-AcDON .......................................................................................... 137

5.4.4.2 DON, 3-AcDON, Cycloheximide .......................................................... 139

5.4.4.3 B-24 V_{H}H Control ............................................................................. 141

5.4.5 V_{H}H Immunolocalization ..................................................................... 141

5.4.6 Mycotoxin Biotransformation Assays ..................................................... 141

5.5 Discussion ...................................................................................................... 145

5.6 Acknowledgements ........................................................................................ 153

5.7 Supplemental .................................................................................................. 154

6 CONCLUSIONS .................................................................................................. 159

7 REFERENCES .................................................................................................... 162
**LIST OF TABLES**

**Table 4.1**  
Nucleotide sequences of primers for PCR amplification of HCAb \( V_H \) genes.................................................................................................91

**Table 4.2**  
HCAb and cIgG fractions of llama serum. Volumes of pre-immune, 42-day and 70-day samples = 12.1, 12.5 and 15.5 mL, respectively.............102

**Table 5.1**  
Sensitivity of *P. pastoris* (KM71H) pPICZB transformants to various ribotoxin treatments tested. Standard deviations (\( \sigma \)) for each mean value shown in brackets.................................................................134

**Table 5.2**  
Mean \( V_H \) intrabody concentration \((t = 0 \text{ h})\) within *P. pastoris* transformants used in cytotoxicity assays.................................................136

**Table 5.S1**  
Raw data and calculations of \( V_H \) intrabody expression in *Pichia pastoris* transformants derived from quantitative Western blot analysis.......155/156

**Table 5.S2**  
HPLC retention times of trichothecene metabolites evaluated after 24 h of growth of *P. pastoris* transformants cultured in YPD media supplemented with 100 \( \mu \text{g.mL}^{-1} \) of highly-purified DON.........................................................158
LIST OF FIGURES

**Figure 1.1** Structure, composition, and molecular weight of trichothecene mycotoxins used within this study........................................................................................................2

**Figure 2.1** Photos of wheat showing progressive signs of FDK (*Fusarium* damaged kernels) in wheat...............................................................6

**Figure 2.2** Disease Cycle of FHB infection of small grain cereals.........................9

**Figure 2.3** The grass floret. A: Partly dissected at anthesis. The ovary and two stigmas comprise the pistil. B: Transverse diagram showing overlap of lemma and palea.......................................................................................12

**Figure 2.4** Scanning electron micrographs of *F. culmorum* colonization of wheat (cv. Agent).................................................................................................................13

**Figure 2.5** Penetration of lemma of wheat (cv. Agent) by *F. culmorum*.................14

**Figure 2.6** Schematic diagram of the first two florets of a wheat spikelet, showing the relationship among the ovary (O), lemma (L), glume (G), palea (P), rachilla (RL), Rachis Node (RN), and Rachis (R) in terms of initial *Fusarium* infection sites (red arrowheads) and systemic infection pathways (blue arrows) of *F. culmorum*.......................................................................................15

**Figure 2.7** Diagram of the general structure, numbering system of the tetracyclic trichothecene nucleus, and specific side-chain groups of selected naturally-occurring trichotheenes of the genus *Fusarium*..................................................20

**Figure 2.8** Structure of DON, 15-AcDON, and 3-AcDON........................................25

**Figure 2.9** Immunogold localization of *Fusarium* toxins in infected host tissue (cv. Agent) by *F. culmorum*...............................................................27

**Figure 3.1** Schematic representation (left) and ribbon diagram (right) of recombinant antibody fragment (Fab)........................................................................39
Figure 3.2  Schematic representation (left) and ribbon diagram (right) of recombinant single chain variable fragment (scFv) antibody.................................40

Figure 3.3  Schematic representation (left) and ribbon diagram (right) of recombinant HCAb variable domain (V_H) of antibody “AMD9”..............................42

Figure 3.4  Schematic representation of a filamentous bacteriophage and its protein coats........................................................................................................................................50

Figure 3.5  Phage (left) and phagemid (right) vectors to construct rAb phage display libraries through the minor coat protein pIII..............................................51

Figure 3.6  Phage display panning cycle...............................................................................................................................53

Figure 3.7  Ribosome display panning cycle...............................................................................................................................56

Figure 4.1  ELISA of polyclonal llama serum response to 250 μg 15-DON-BSA conjugate...............................................................................................101

Figure 4.2  Amino acid sequence of NAT-267 V_HH from fourth round of panning..104

Figure 4.3  FP inhibition of mNAT-267 and pNAT-267 V_HH binding and 56-day polyclonal llama serum binding to DON-fluorescein tracer with DON toxin (A); 3-AcDON (B); and 15-AcDON (C) from 5 μg·mL⁻¹ to 9.8 ng·mL⁻¹.................................................................107

Figure 4.4  Overlay sensorgrams of mNAT-267 V_HH steady state (Req) binding to 15-DON-HRP conjugate..................................................................108

Figure 4.5  (A) Steady state (Req) affinity binding (RU) mNAT-267 V_HH to 15-DON-HRP conjugate for ten monomer concentrations, as shown in Figure 4.4. (B) Scatchard plot of steady state ‘Req’ binding (RU) relative to Req / C (mNAT-267 V_HH concentration) (RU/μM).........................................................109

Figure 4.6  Structure, composition, and molecular weight of trichothecene mycotoxins used in this study.................................................................114
Figure 5.1  Structure, composition, and molecular weight of trichothecene mycotoxins used in this study..........................................................122

Figure 5.2  Diagram of pPICZB expression vector (3745 bp) with V<sub>H</sub>H gene (NAT-267 = 387 bp, B-24 = 390 bp). ..........................................................125

Figure 5.3  Quantitative Western blot analysis of V<sub>H</sub>H intrabody expression within P. pastoris cytotoxicity assays........................................135

Figure 5.4  Cytotoxicity assay of P. pastoris pPICZB and NAT-267 V<sub>H</sub>H transformant growth measured by optical density at 620 nm (OD<sub>620</sub>) to 15-AcDON at doses of 20 to 200 µg·mL<sup>-1</sup> ..........................................................138

Figure 5.5  Cytotoxicity assay of P. pastoris pPICZB and NAT-267 V<sub>H</sub>H transformant growth measured by optical density at 620 nm (OD<sub>620</sub>) to DON and cycloheximide at doses of 50 to 200 µg·mL<sup>-1</sup> ..........................................................140

Figure 5.6  Cytotoxicity assays showing the effect of various concentrations (50 to 200 µg·mL<sup>-1</sup>) of 15-AcDON (A) and cycloheximide (B) on the growth of P. pastoris pPICZB and non-specific B-24 V<sub>H</sub>H transformants as measured by optical density at 620 nm (OD<sub>620</sub>) ..........................................................142

Figure 5.7  Representative confocal microscopy photographs of P. pastoris (KM71H) cells immuno-probed with anti-HA epitope mAb-FITC conjugate and DAPI stain. Transformants expressing pPICZB empty vector (control) (A) and NAT-267 V<sub>H</sub>H intrabody (B) ..........................................................143

Figure 5.8  Representative 500 mHz ¹H NMR spectrum of A = 15-AcDON Standard. B = HPLC fraction of P. pastoris cell lysate sample (24 hrs) ...............144

Figure 5.S1  Homology chart of DNA and amino acid sequence of codon-optimized NAT-267 and control B-24 V<sub>H</sub>H fragments........................................154
1 INTRODUCTION, OBJECTIVES, AND HYPOTHESIS

*Fusarium* head blight (FHB) of cereals is caused by morphologically similar species (*F. graminearum*, *F. culmorum*, etc.) common throughout global agricultural regions. With few exceptions, *Fusarium* epidemics are characterized by cyclical and highly aggressive infection of cereal crops with economic impacts on food and feed industries that are immediate and far reaching (Demcey Johnson et al., 2003; McMullen, 2003; Miller, 2008).

Trichothecene mycotoxins are a toxin class commonly found within agricultural commodities infected by *Fusarium*. Trichothecene toxins represent a large group of >180 sesquiterpenoids that have molecular weights typically ranging from 200-500 Da and are characterized by a tricyclic ring structure containing a double bond at C-9,10 and an epoxide group at C-13 (reviewed in Desjardins, 2006; Foroud and Eudes, 2009; Mirocha et al., 2003). Regardless of size and structural composition, trichothecenes are potent inhibitors of eukaryotic protein synthesis with specific activity on ribosomal protein L3 (RPL3) within the 60S subunit resulting in inhibition of peptidyl transferase activity (Carter and Cannon, 1977; Rocha et al., 2005). Although the capacity to inhibit protein synthesis is regarded as central to trichothecene cytotoxicity (Ueno, 1984; Rotter et al., 1996), adverse effects on eukaryotic cells may actually be attributed to dysregulation of cellular signaling and alterations in downstream gene expression (Pestka, 2008).

Deoxynivalenol (3,7,15-trihydroxy, 12,13-epoxy-trichothece-9-en-8-one), or DON, is one of many members of the trichothece group of mycotoxins produced by *Fusarium* species. DON is a low molecular weight hapten (MW = 296 Da) containing one primary and two secondary hydroxyl groups conferring limited solubility in water and high
solubility in various polar solvents such as methanol and acetonitrile (reviewed in Desjardins, 2006; Mirocha et al., 2003). Of the trichothecene mycotoxins that accumulate within cereal grain, DON and its closely related-acetylated analogues (i.e., 15-Acetyl-Deoxynivalenol (15-AcDON) and 3-Acetyl-Deoxynivalenol (3-AcDON)) (Figure 1.1) are considered among the most common and widely distributed food and feed contaminants (Abramson et al., 2001; D’Mello et al., 1999; Greenhalgh et al., 1986; Snijders, 1990a).

![Chemical Structures of DON, 15-AcDON, and 3-AcDON](image)

<table>
<thead>
<tr>
<th>Trichothecene</th>
<th>MW(^a) (Da)</th>
<th>Oxygenation and Esterification at Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Acetyl-DON</td>
<td>338</td>
<td>O-CO-CH(_3)</td>
</tr>
<tr>
<td>15-Acetyl-DON</td>
<td>338</td>
<td>OH</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>296</td>
<td>OH</td>
</tr>
</tbody>
</table>

\(^a\) MW = Molecular Weight

**Figure 1.1** Structure, composition, and molecular weight of DON, 15-AcDON, and 3-AcDON (Desjardins, 2006).

There is a demonstrated correlation between *in planta* DON accumulation and *Fusarium* virulence in susceptible cultivars of wheat (Mesterházy, 2002) and maize (Harris et al., 1999a). Based on these findings, mechanisms which convey innate and
acquired host plant resistance to DON and other trichothecene toxins have received considerable attention. To date, *in planta* trichothecene resistance has been achieved through mechanisms that alter targeted proteins within host cell ribosomes (e.g., Harris and Gleddie, 2001; Mitterbauer et al., 2004), promote metabolic transformation to less toxic forms: e.g., DON-glucosyl conjugate (Poppenberger et al., 2003) or to 3-AcDON (Alexander, 2008), and/or reduce intracellular concentrations to effectively limit mycotoxin exposure to sensitive cellular targets. Collectively, such research can be applied to assess and impart novel mechanisms of trichothecene resistance within eukaryotic test systems such as yeast or *Arabidopsis* (Mitterbauer and Adam, 2002) for eventual application in higher order plants such as cereals and maize.

Current applications of antibody (Ab)-based technology involving FHB and trichothecene toxins such as DON, 3-AcDON, and 15-AcDON are generally limited to immunoassays based on polyclonal and monoclonal antibodies (mAb) to detect mycotoxins within grain, food, feed, and animal serum (e.g., Maragos and McCormick, 2000; Nicol et al., 1993; Sinha et al., 1995). Although these tests provide robust and precise results, mAb development requires highly specialized equipment and labor-intensive procedures to select, culture, and maintain optimal hybridoma cell lines. Because of these limitations, recombinant Ab and phage display technologies are often used to generate target-specific recombinant antibody (rAb) fragments from Ab cell lines. In this thesis, these two technologies were used to develop and quantify the efficacy of a single-domain heavy chain antibody fragment (i.e., $V_{\text{H}}H$) with affinity for 15-AcDON.
V_{H}H fragments are derived from the camelidae heavy chain IgG subfamily and are among the smallest functional rAb fragments known. V_{H}H fragments exhibit the same exquisite specificity as larger immunoglobulins, and confer added biochemical advantages of high solubility, stability, and robust expression in various recombinant systems (Holliger and Hudson, 2005; Muyldermans, 2001). V_{H}H fragments have been generated against a wide variety of epitopes and antigen (Ag) types including low molecular weight ligands (haptens) (Nguyen et al., 2000; Sheedy et al., 2006; van der Linden et al., 2000) and toxins (e.g., Doyle et al., 2008).

The advantages associated with ease of expression, stability, and solubility of V_{H}H fragments can be attributed to key differences when compared to standard rAb fragments (e.g., scFv, Fv, Fab, etc.). V_{H}H fragments are small (~ 14 – 15 kDa), typically have hydrophilic amino acid substitutions within the second framework region (FR-2), and by their single-domain nature, do not require the linker associated with the standard scFv format which can lead to problems associated with aggregation, proteolytic degradation, and variable expression within recombinant hosts (Arbabi-Ghahroudi et al., 2005). Improved functionality over various temperature and solvent regimes (Goldman et al., 2006; van der Linden et al., 1999) coupled with high levels of recombinant expression combine to make V_{H}H fragments useful reagents within next-generation immunoassays (Goldman et al., 2006, Ladenson et al., 2006). When expressed as intrabody fragments within recombinant hosts, V_{H}H fragments can be used as Ag-specific reagents to elucidate potential in vivo mechanisms of immunomodulation with applications ranging
from pharmacology (Holliger and Hudson, 2005; Saerens et al., 2008) to plant science (Jobling et al., 2003).

The research summarized within this Ph.D. project was based on two related objectives. Initial experiments were established to identify, characterize, and assess binding affinity of a trichothecene-specific recombinant V_{H}H fragment that was isolated from a hyper-immunized phage-display library. The second group of experiments were focused on an *in vivo* assessment of a V_{H}H intrabody immunomodulation of the cytotoxic effects of the target mycotoxin within a model eukaryotic system based on *Pichia pastoris*. Collectively, this research was established as a ‘proof of concept’ model to demonstrate that intrabody expression, i.e. expression of recombinant V_{H}H fragments, could impart a novel means of mycotoxin-specific resistance in susceptible eukaryotic organisms.

**Hypothesis**

*Expression of a mycotoxin-specific V_{H}H antibody fragment within a model eukaryotic test system will limit mycotoxin-specific cytotoxicity and inherently reduce disease symptoms associated with FHB pathogenesis.*
2 LITERATURE REVIEW 1: FUSARIUM HEAD BLIGHT AND TRICHOТЕCENE MYCOTOXINS: NOVEL TARGETS FOR RECOMBINANT ANTIBODY TECHNOLOGY

2.1 Introduction

*Fusarium* head blight (FHB) of small grain cereals is a fungal disease caused by a range of *Fusarium* species common throughout the world’s agricultural regions. Epidemics are characterized by highly cyclical and aggressive plant pathogenesis with few known mechanisms of control. Despite causing significant yield loss, the principal economic impact of a FHB epidemic usually results in harvested grain with severely reduced quality since *Fusarium*-damaged kernels (FDK) ([Figure 2.1](#)) are often shrunken and shriveled with little economic value. Reduced seed quality and value is exacerbated by the fact that infected grain is often contaminated with toxic fungal metabolites, or mycotoxins, which affect food and feed quality (Bai and Shaner, 1994; Dickson, 1942; Foroud and Eudes, 2009; McMullen, 2003; Miller, 2008; Parry et al., 1995; etc.).

![Figure 2.1](#) Photos of wheat showing progressive signs of FDK (*Fusarium* damaged kernels) in wheat (Harvest samples from Syngenta Field Trials, 2004).

The two major classes of mycotoxins common to *Fusarium* infected small grain cereals are trichothecenes and zearalenone. Trichothecenes are tricyclic sesquiterpenes that contain a 12,13-epoxide ring and a double bond between carbons 9 and 10.
Zearalenones are an unrelated group of *Fusarium* mycotoxins derived by different cyclizations and modifications of nonaketide precursors. Like trichothecenes, zearalenones are low molecular weight, heat-stable mycotoxins commonly found within *Fusarium*-infected cereals and maize (Desjardins, 2006).

Unlike zearalenones, which are not generally considered to be phytotoxic compounds, trichothecenes can produce a wide array of phytotoxic effects within plant tissue. Some of the first evidence to link trichothecenes as a mechanism of virulence for *Fusarium* infection of plant tissue was provided by gene disruption experiments. Various researchers showed that loss-of-function mutations in the *Tri5* gene (key enzyme within the trichothecene biosynthesis pathway) within virulent *Fusarium* spp. resulted in reduced fungal pathogenesis and colonization of plant tissue (Desjardins et al., 1996; Eudes et al., 2001; Proctor et al., 1995). Subsequent research, as outlined within this chapter, demonstrated that various other techniques to limit trichothecene (particularly deoxynivalenol) phytotoxicity could limit *Fusarium* infection of small grain cereals and maize. In addition, research has shown that the production of DON within plant tissue is commonly associated with the necrotrophic stage of *Fusarium* pathogenesis during which host-plant tissue dies as the fungus advances and proliferates within infected cereal tissue (Mesterházy, 2002; Muehlbauer and Bushnell, 2003; Proctor et al., 2002).

The following chapter provides an overview of the general aspects related to the occurrence of, and control strategies associated with, FHB infection and trichothecene contamination of agricultural crops. Given the vast array and enormous scope of publications covering this subject matter, this introductory chapter is intended to be a
concise and general overview to support, and align with subsequent research chapters within this thesis.

2.2  *Fusarium Head Blight*

FHB has the most dramatic and widespread effect on cereal production during major epidemics; however once established within an area, even at baseline infestation levels, FHB can be a major limitation to cereal production (Diaz de Ackermann and Kohli, 1997a; Mesterházy, 1997; Sutton, 1982; Wang, 1997). Arguably, the best documented, and perhaps the most costly FHB epidemic in North America occurred in the upper Great Plains of the USA and within the provinces of Manitoba, Ontario, and Quebec, Canada between 1993 and 1998 (Gilbert et al., 1994; McMullen, 2003; McMullen et al., 1997; Windels, 2000; etc.). Estimated economic losses associated with lost crop yield and reduced harvest quality over the course of the epidemic range from $1.3 to nearly $3.0 billion USD (Demcey Johnson et al., 2003; Windels, 2000).

2.2.1  *Disease Cycle*

The critical factors or conditions which must exist for FHB to occur are described by the classic plant pathologist ‘Disease Triangle’ denoted by: 1.) inoculum, 2.) susceptible host, and 3.) the right mix of environmental conditions (Shaner, 2003). More specifically, the establishment of FHB can be attributed to the release of inoculum from partially-degraded crop residues throughout periods of warm, windy, humid weather during anthesis (i.e. flowering) of susceptible host crops (*Figure 2.2*).

FHB is a monocyclic disease that is initiated by inoculum carried within air currents or splashing water (Sutton, 1982). The quantity of primary inoculum therefore directly
influences the intensity and extent of disease development during infection (Bai and Shaner, 1994; Khonga and Sutton, 1986, 1988). Although secondary infections are theoretically possible, the monocyclic nature of FHB can be attributed to the relatively short period of crop susceptibility and limited window of favorable environmental conditions which favor FHB establishment (Fernando et al., 1997; Shaner, 2003).

Figure 2.2 Disease Cycle of FHB infection of small grain cereals (from Bergstrom, 1993).

_Fusarium_ seedling blight, or _Fusarium_ crown and foot rot, is a related seed-borne disease of cereals (Figure 2.2). Affected seedlings emerge stunted and yellow with the crown, roots and/or lower stem having a brown to red-brown appearance (Sutton, 1992). Although FHB is not regarded as a systemic disease based on an infection that develops from _Fusarium_ infected seed, seedling blight may serve as a source of secondary inoculum within the canopy of the growing crop (Parry et al., 1995).
2.2.1.1 Inoculum

While several species of *Fusarium* cause FHB around the world, including *F. graminearum* Schw. (Teleomorph – *Gibberella zeae* Schw. Petch), *F. culmorum* (Wm. G. Smith) Sacc., and *F. avenaceum* (Corda ex Fr.) Sacc. (Teleomorph - *G. avenacea* (Cook)), etc., *F. graminearum* is the most common within North America, Europe and Asia (McMullen et al., 1997; Parry et al., 1995; Sutton, 1982; Tekauz et al., 2000; Wilcoxson et al., 1988).

Infected crop residue (i.e., debris of previously-harvested wheat, barley, maize) is the principle source of primary *F. graminearum* inoculum for FHB infestations (Figure 2.2). Therefore, inoculum levels are related to the amount of crop residue and the degree to which crop debris is infected on the surface of soil (Khonga and Sutton, 1988: Sutton, 1982). Challenges associated with inoculum build-up are exacerbated by reduced tillage practices, which result in larger amounts of, and longer degradation profiles for, crop residues within the top layer of soil (Shaner, 2003). Primary types of inoculum of virulent *Fusarium* species include ascospores (*F. graminearum*), as well as conidia, hyphal fragments, and chlamydospores (*F. culmorum*, *F. avenaceum*, etc.). Environment has a direct impact on inoculum production and release since warm, moist, and windy weather disseminate *Fusarium* inocula by rain-splash and air currents to enable direct contact of sexual and asexual inoculum with susceptible tissue of host plants (Menzies and Gilbert, 2003) (Figure 2.2).
2.2.1.2 Environment

The role of environment in FHB epidemiology is a complex subject that has been extensively reviewed (Andersen, 1948; Sutton, 1982; Parry et al., 1995; Shaner, 2003). The inherent complexity of the disease is associated primarily with temperature and duration of continuous moisture, which are both tied to rainfall and prevailing humidity levels and are inherently variable factors that affect both inoculum production and dispersal as well as the duration of host crop susceptibility to Fusarium infection. Therefore, the establishment of the FHB disease cycle (Figure 2.2) is dependent upon prolonged precipitation and/or periods of high humidity (e.g., 92 - 94% R.H.) in conjunction with warm temperatures during anthesis of host crops (Sutton, 1982). Prevailing moisture has a greater role than temperature in terms of inoculum production, distribution, and colonization and pathogen development within infected heads because favorable temperatures for FHB establishment are generally common when cereal crops are flowering (Shaner, 2003; Sutton, 1982). However, it is also well established that longer periods of prevailing moisture are required for FHB infection when temperatures are lower or higher than 25°C (Andersen, 1948).

2.2.1.3 Host Crop

The initial point of Fusarium infection associated with FHB of cereals is fungal colonization of floret and caryopsis tissue. Cereal florets are composed of an inner palea (Figure 2.3A) and an outer lemma (Figure 2.3B), which overlap and partially enclose the edge of the palea. Together, the lemma and palea enclose the pistil, stamens, lodicules, and an ovary to form the caryopsis (Figure 2.3) (Bushnell et al., 2003).
Floret and caryopsis host tissues are most susceptible to *Fusarium* infection within a 3 - 5 day period corresponding to flowering or anthesis (i.e., BBCH = 61 - 69). However, depending on prevailing environmental conditions and inoculum levels, colonization can occur from mid inflorescence (i.e., BBCH = 55) to the soft dough growth stage (i.e., BBCH = 85) (Paulitz et al., 1997). After fungal colonization, disease severity progresses rapidly to a necrotrophic systemic infection which results in widespread *Fusarium* infection throughout the cereal spike (Shaner, 2003).

**Figure 2.3** The grass floret/caryopsis. **A**: Partly dissected at anthesis. The ovary and two stigmas comprise the pistil. **B**: Transverse diagram showing overlap of lemma and palea (adapted from Bushnell et al., 2003).
2.2.2 Pathways of FHB Pathogenesis

2.2.2.1 Fungal Colonization

Upon initial germination of ascospores (i.e., first ~ six hours), *Fusarium* hyphae rapidly elongate over the abaxial (exterior) surfaces of glumes and florets to form a complex mycelial network across the surface of colonized host tissue (Figure 2.4A).

![Figure 2.4 Scanning electron micrographs of *F. culmorum* colonization of wheat (cv. Agent). A, Hyphal network between pollen grains (PG) on brush hairs at the top of the ovary (48 hours post infection (HPI)). B, Hyphae (H) within digested host cuticular layer on the inner surface of palea next to plant hair structure (48 HPI). C, Hyphae (H) growing upward over the edge of the glume from germinating macroconidia (M). (Kang and Buchenauer, 2000).](image)

From initial stages of infection, hyphae initiate penetration of host tissue through enzymatic degradation of abaxial surfaces of the caryopsis (Baláza and Bagi, 1997; Urbanek, 1989; etc.) (Figure 2.4B). Seventy-six HPI, hyphae can progress across the outer surfaces of the lemma and glume to reach the developing caryopsis (Figure 2.4C).
Throughout the later stages of this biotrophic infection process, mycelial growth progresses to the point where fungal reproductive structures (conidiophores) are established on the surface of infected tissue (Pritsch et al., 2000).

_Fusarium_ hyphae follow various routes to reach interior surfaces of infected plant tissue (Bushnell et al., 2003). These routes of entry include direct wall penetration of the epidermal cell wall by a hyphal peg (Figure 2.5A), entry through stomates (Figure 2.5B), or colonization of openings between lemma and palea during the brief period of dehiscence when cereal florets are open (Lewandowski and Bushnell, 2001). Following primary penetration, _Fusarium_ hyphae exhibit a subcuticular growth pattern with flat, branched hyphae growing between the cuticle and epidermal cell walls of host tissue (Pritsch et al., 2000).

**Figure 2.5** Penetration of lemma of wheat (variety = Agent) by _F. culmorum_. A, Transmission electron micrograph (TEM) showing penetration peg (arrowhead) from an infection hyphae (IH) invading epidermal cell wall (EW) at 36 HPI. B, Scanning electron micrograph (SEM) showing growth of hyphae (H) through a stomatal (S) opening on the inner surface of the lemma 48 HPI (Kang and Buchenauer, 2000).
2.2.2.2 Systemic Infection

Once fungal colonization is established, *Fusarium* hyphae infect adjacent florets on the same spikelet through a systemic and necrotic infection of the rachis and rachis node (Figure 2.6). Systemic infection is marked by increased fungal colonization and a rapid necrotic deterioration of host-cell tissue because invasive hyphae progress from floret to floret (Ribichich et al., 2000; Schroeder and Christensen, 1963). Thereafter, infected florets cease to develop as host cell tissue and organelles become completely fragmented as hyphae spread vertically and horizontally within the rachis and rachis node (Figure 2.6).

Figure 2.6 Schematic diagram of the first two florets of a wheat spikelet, showing the relationship among the ovary (O), lemma (L), glume (G), palea (P), rachilla (RL), rachis node (RN), and rachis (R) in terms of initial *Fusarium* infection sites (red arrowheads) and systemic infection pathways (blue arrows) of *F. culmorum*. Dotted lines and dotted areas indicate vascular tissues within the RN (Kang and Buchenauer, 2000).
Continued necrotic infection leads to a degeneration of plant cytoplasts, cell walls, chloroplasts and other plastids, which limit the ability of the phloem and xylem to deliver nutrients to developing tissue. FHB infection is characterized by premature death of infected spikelets, resulting in bleached and shriveled kernels often referred to as tombstones or *Fusarium* damaged kernels (Figure 2.1).

### 2.2.3 FHB Control Strategies

#### 2.2.3.1 Agronomic and Cultural Practices

FHB epidemics are initiated by inoculum (mostly ascospores for *F. graminearum*) produced within crop residues on the soil surface. Any measure to reduce crop residue accumulation and longevity on the soil surface such as plowing or burning excess residue has the potential to reduce primary inoculum levels (Dill-Macky and Jones, 2000; Fernandez et al., 1993). Furthermore, agronomic techniques to stagger seeding dates and maintain long rotation periods between susceptible crops (e.g. cereals or maize) within a given area are also recommended practices to reduce inoculum levels and limit FHB development (Dill-Macky and Jones, 2000; McMullen et al., 1997).

Unfortunately, it is difficult to predict the effectiveness of such practices to limit FHB (Meier et al., 2000) as disease establishment within a susceptible crop is highly dependent on climate and irregular in terms of disease incidence and severity. In many cases, environmental conditions associated with the year of production tend to be the single largest factor associated with variation in disease impact (Schaafsma et al., 2001).
2.2.3.2 **Fungicides**

Fungicide applications are commonly used to limit yield losses associated with FHB infection and mycotoxin contamination of small grain cereals (reviewed by Mesterházy, 2003b). Among the currently-available fungicides registered for FHB control and mycotoxin suppression, products based on triazole chemistry (e.g., tebuconazole, metconazole, or prothioconazole) are considered to be the most efficacious (Jennings, 2002; Mesterházy, 2003b; etc.). However, commercial experience and extensive trial data from various sources have shown that fungicide efficacy is variable at best (e.g., D’Mello et al., 1998; Homdork et al., 2000; Jennings et al., 2000; Simpson et al., 2001). Chemical disease control is dependent on timing and method of application, host crop FHB susceptibility, as well as prevailing environmental conditions, and *Fusarium* inoculum levels (Magan et al., 2002; Mesterházy, 2003b). Given the complexity associated with all of the variables that affect FHB pathogenesis, fungicide treatments are, therefore, recommended and considered most effective when used as part of an integrated crop management strategy (Milus and Parsons, 1994; Teich, 1989) that is based on an accurate disease forecasting system and sound agronomy (McMullen et al., 1997; Schaalma et al., 2001).

2.2.3.3 **Biological Control Agents**

A common approach to biological control strategies is to apply non-pathogenic organisms in advance of *Fusarium* infection of cereals as part of a competitive exclusion strategy to disrupt the FHB disease cycle upon spikelet colonization and infection (reviewed by da Luz et al., 2003). Numerous research groups have tested a wide range of
biological control agents against diseases attributed to *Fusarium* spp. (e.g., Huang et al., 1993; Kempf and Wolf, 1989; Shi and Wang, 1991; Stockwell et al., 2000; etc.); however, very few researchers have reported successful large-scale applications of this strategy. Additional work and screening is required to identify biological control strains and application technologies to limit FHB colonization and mycotoxin accumulation under various field conditions (Chen et al., 2000; da Luz et al., 2003).

### 2.2.3.4 Crop Breeding

The standard approach to develop cultivars with enhanced FHB resistance has been to cross cultivars with resistance or enhanced tolerance to *Fusarium* infection with a susceptible, but agronomically superior and locally adapted, cultivars or crop varieties to generate progeny with a favorable phenotype. Unfortunately, progress to date in developing cereal cultivars with enhanced resistance to FHB-related yield loss and mycotoxin contamination has been limited (reviewed in: Bai et al., 2003; Miedaner, 2002; Mesterházy, 2003a; Steffenson, 2003). Lack of progress using classical plant breeding efforts has been attributed to: i) limitations associated with practical techniques to evaluate FHB resistance within large breeding programs, ii) difficulties in establishing consistent levels of *Fusarium* inoculum within FHB nurseries on a cost-effective basis, iii) limited availability of cereal germplasm with high levels of innate resistance, and iv) inconsistent financial support for public research (Dill-Macky, 2003; Sutton, 1982).

Sources of resistance to FHB in wheat are based on spring wheat genotypes with innate resistance. Varieties such as: ‘Sumai 3’, ‘Nobeoka Bozu’, ‘Shanghai 7-31B’, ‘Nyubai’, ‘Ning 7840’, and ‘Frontana’ are commonly use in breeding programs around
the world to develop regionally-adapted cultivars with enhanced FHB and mycotoxin resistance (Buerstmayr et al., 1996; Diaz de Ackermann and Kohli, 1997b; Gilchrist et al., 1997; Snijders, 1990b; etc.). Relatively few barley cultivars have been identified with a high level of innate resistance to FHB and mycotoxin accumulation (Steffenson, 2003). Unfortunately, most accessions from *Fusarium*-resistant varieties exhibit unfavorable agronomic traits which render them largely unsuitable for widespread adoption within FHB affected regions (Chen et al., 2000). Furthermore, most new accessions produced by backcrossing resistant varieties with regionally-adapted germplasm tend to have limited and often unstable FHB resistance (Mesterházy, 2003a).

### 2.2.4 Classifications of FHB Resistance

Classic descriptions of host-plant resistance to initial infection and to spread of *Fusarium* within the spike were initially categorized as ‘Type 1’ and ‘Type 2’, resistance, respectively (Schroeder and Christensen, 1963). Type 1 resistance is regarded as an important component of head blight resistance of barley, since disease resistance given by percentage of florets infected is inherently more important than spread from floret to floret. Type 2 resistance is believed to be more important for wheat as FHB tends to spread more readily from spikelet to spikelet (Bushnell et al., 2003).

Since the development of this classification system, three other types of resistance have been postulated; ‘Type 3’ resistance, which is based on an ability to limit accumulation of trichothecene toxins in kernels (Miller et al., 1985), ‘Type 4’ resistance based on kernel infection (Mesterházy, 1995), and ‘Type 5’ resistance based on increased tolerance to yield loss in the presence of FHB (Mesterházy, 1995). Unfortunately, beyond
the descriptions of Types 1 and 2, there is little agreement on the order and general
designation of various mechanisms of FHB resistance in cereals.

2.3 Trichothecene Mycotoxins

Trichothecenes are low molecular weight sesquiterpenoid mycotoxins characterized
by a tricyclic ring structure with a double bond at C-9,10 and an epoxide group at C-12,13
(Mirocha et al., 2003) (Figure 2.7).

![Diagram of the general structure, numbering system of the tetracyclic
trichothecene nucleus, and specific side-chain groups of naturally occurring
trichothecenes of the genus Fusarium (Desjardins, 2006).]

<table>
<thead>
<tr>
<th>Trichothecene</th>
<th>MW</th>
<th>Oxygenation and Esterification at Position&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-2 Toxin</td>
<td>466</td>
<td>-OH -OAC -OAC -H -Olsoval</td>
</tr>
<tr>
<td>HT-2 Toxin</td>
<td>424</td>
<td>-OH -OH -OAC -H -Olsoval</td>
</tr>
<tr>
<td>4,15-Diacetoxyscripenol</td>
<td>366</td>
<td>-OH -OAC -OAC -H -H</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>312</td>
<td>-OH -OH -OH -OH =O</td>
</tr>
<tr>
<td>DON</td>
<td>296</td>
<td>-OH -H -OH -OH =O</td>
</tr>
<tr>
<td>15-AcDON</td>
<td>338</td>
<td>-OH -H -OCH&lt;sub&gt;3&lt;/sub&gt; -OH =O</td>
</tr>
<tr>
<td>3-AcDON</td>
<td>338</td>
<td>-OCH&lt;sub&gt;3&lt;/sub&gt; -H -OH -OH =O</td>
</tr>
</tbody>
</table>

<sup>a</sup> MW = Molecular Weight (Daltons).
<sup>b</sup> OAc (Acetyl Ester = -O-CO-CH<sub>3</sub>), Olsoval (Isovalerate Ester = -OCOCH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>).

Figure 2.7 Diagram of the general structure, numbering system of the tetracyclic
trichothecene nucleus, and specific side-chain groups of naturally occurring
trichothecenes of the genus *Fusarium* (Desjardins, 2006).

Type A and B structural classes can be grouped based on type of substituent group
at C-8 (Chelkowski, 1989; Desjardins, 2006; Foroud and Eudes, 2009; Mirocha et al.,
2003; Perkowski et al., 1995). Type A trichothecenes (e.g., T-2 toxin, HT-2 toxin, diacetoxyscirpenol) have hydrogen, hydroxyl, or ester groups at C-8 position. Type B trichothecenes (e.g., nivalenol, DON, 15-AcDON) have a keto substituent at C-8 (Figure 2.7).

The name denoted to trichothecene mycotoxins is derived from the fungus *Trichothecium*, which produces the natural product called trichothecin (Freeman and Morrison, 1948). In pure crystalline form, trichothecenes tend to be colourless, odourless, relatively soluble in polar solvents, and very stable within a wide range of matrices (Scott, 1991; Trenholm et al., 1991; 1992).

### 2.3.1 Mode of Action

The biochemical mode of action and cytotoxic effects of trichothecene mycotoxins within eukaryotic cells has been extensively reviewed (e.g., Canady et al., 2001; Desjardins, 2006; Prelusky et al., 1994; Rotter et al., 1996; Ueno, 1983). In general, trichothecene cytotoxicity is attributed to the disruption of peptidyl-transferase activity given by mycotoxin binding to ribosomal protein L3 (RPL3) within the 60S subunit of eukaryotic ribosomes (Feinberg and McLaughlin, 1989; McLaughlin et al., 1977).

Although binding to RPL3 has been specifically attributed to the presence of the C-12,13 epoxide group common to all trichothecenes (Figure 2.7) (Carter and Cannon, 1977; Rocha et al., 2005; Rotter et al., 1996), the number and position of substituent groups on the tetracyclic nucleus (Figure 2.7) has a profound influence on the relative toxicity of various trichothecene mycotoxins. Given the inherent diversity of the various side chain substituents that comprise both Type A and B trichothecenes, a wide range of
physico-chemical properties and cytotoxic effects of trichothecenes has been exhibited on various types of eukaryotic cells (Desjardins, 2006; Desjardins and Hohn, 1997; Kuiper-Goodman, 1994; Mirocha, 1983; Nishiuchi et al., 2006; Ohtsubo, 1983; Otokawa, 1983; Prelusky et al., 1994; Terao, 1983; Ueno, 1983; etc.).

The presence of different side chains on the trichothecene nucleus is, therefore, a key determinant of relative cytotoxicity values and specific cytotoxic effects for trichothecene mycotoxins. Depending on the test system, different substituents can lead to diverse effects including inhibition of protein synthesis during translation, transcription, or elongation as well as various secondary effects such as inhibition of DNA and RNA synthesis, necrosis and inhibition of cell division (Feinberg and McLaughlin, 1989), inhibition of mitochondria enzymes, immunotoxicity (Creppy, 2002; Sudakin, 2003).

2.3.2 Mechanisms of Fungal Protection

Although trichothecenes have no function related to the growth of the producing organism, they are involved in the biological adaptation and ecology of *Fusarium* species to various environments (Desjardins, 2006). Part of this adaptation mechanism involves various specialized genetic adaptations by the producing organism during the biosynthesis of trichothecenes as a means of ‘self-preservation’ (Cundliffe, 1989).

Genes that encode for detoxification mechanisms and/or molecular efflux pumps to limit cytotoxicity to the parent organism are believed to be the principal means by which virulent *Fusarium* species protect themselves from trichothecone-specific cytotoxicity. For example, the *F. sporotrichioides* gene *Tri12*, which encodes an efflux pump, is
responsible for pumping trichothecenes out of, and away from, the fungal cell (Alexander et al., 1999). Genes to help limit the metabolic cytotoxicity of trichothecenes have also been demonstrated as a defense response within *Fusarium* species. For example, the gene *Tri101* encodes an acetyl-transferase enzyme responsible for the addition of an acetyl-ester side-chain group (R-OHCH₃) to trichothecene mycotoxins such as DON as a mechanism to reduce molecular cytotoxicity to the parent organism (Kimura et al., 1998b).

### 2.3.3 Effects on Plants

The toxicity of trichothecenes to plant cells was first reported in 1961 (Brian et al., 1961). Since this initial work, trichothecene toxins are well established as phytotoxic compounds that produce wilting, chlorosis, necrosis, and various other non-specific effects within plant tissue (Cutler and Jarvis, 1985, Desjardins, 2006; Nishiuchi, et al., 2006; Packa, 1991; Wakulinski, 1989, etc.). Inhibition of ribosomal protein synthesis is a common mechanism of phytotoxicity; however, some trichothecenes affect electron transport chains, cellular membranes, leaf chlorophyll synthesis and other functions within plant tissue. However, many aspects related to the specific effects of trichothecenes within plant systems, particularly host cell defense mechanisms, remain unknown (Desjardins, 2006).

Genetic disruption techniques to produce ‘knock-out’ strains of virulent *Fusarium* species have been invaluable tools to clarify the relationship between *Fusarium* pathogenicity and trichothecene production within host plant cell tissue. Desjardins et al. (1989) demonstrated that inoculation of parsnip root discs with a T-2 producing wild type
strain of *F. sporotrichioides* caused extensive root rot, while the *Tri4* mutant, which produced little or no trichothecene, caused significantly less root tissue damage. Similar studies conducted with a mutant strain of *F. graminearum*, where the trichodiene synthase (*Tri5*) gene was altered to impair trichothecene biosynthesis, were initiated to examine the effect of trichothecene production on seedling emergence and height of *Fusarium*-inoculated wheat, maize, oat and rye (Proctor et al., 1995). Relative to trichothecene producing (*Tri5*) wild-type strains, non-trichothecene producing (*Tri5*) mutant strains had reduced incidence and severity of disease (i.e., bleaching of heads, yield reduction, etc.) and reduced fungal spread from single spikelet inoculations (Desjardins et al., 1996; Mirocha et al., 1997; Proctor et al., 1995). Similar results were noted by Harris et al. (1999a) who found that mutant strains of *F. graminearum*, which did not produce trichothecene, were less virulent on inoculated maize. A comprehensive summary of these as well as subsequent *Tri5* disruption studies provided by Desjardins (2006) clearly demonstrates that trichothecenes act to enhance the ability of *Fusarium* to cause disease on susceptible host plants.

### 2.4 DON, 3-AcDON, and 15-AcDON

Most phytopathogenic *F. graminearum* species are believed to produce 15-AcDON or 3-AcDON as labile, acetylated precursors to DON (Figure 2.8). Production of either acetylated compound is geography-dependent since 3-AcDON chemotypes tend to dominate in Asia and Europe, while *Fusarium* species which produce 15-AcDON have been generally more prevalent in North America (Goswami and Kistler, 2004; Moss and Thrane, 2004). Therefore, the accumulation of DON within plant tissue is believed to be
a metabolic process conferred by fungal and plant carboxyl-esterases, which continuously deacetylate either 15-AcDON or 3-AcDON within plant tissue (Evans et al., 1997; Mitterbauer and Adam, 2002; Scott et al., 1984).

![Chemical Structures](image)

**Figure 2.8** Structure of DON, 15-AcDON, and 3-AcDON (Desjardins, 2006).

### 2.4.1 Contamination of Food and Feed

After FHB infection of cereals, DON, 15-AcDON and 3-AcDON accumulate within grain as thermally stable and persistent contaminants through storage, handling and processing of food and feed (Canady et al., 2001; Desjardins, 2006; D'Mello et al., 1999, Visconti and De Girolamo, 2002). Therefore, DON and either 3-AcDON or 15-AcDON are considered food- and feed-borne contaminants that are hazardous to human and animal health (reviewed in Canady et al., 2001; D'Mello et al., 1999; Desjardins, 2006).

The effects of chronic exposure to DON within small-grain cereals has been widely tested within domestic livestock. Among animals tested, swine are the most sensitive to DON exposure (Trenholm et al., 1994). In fact, swine producers termed DON “vomitoxin” when they associated observed emesis (vomiting) following the
consumption of *Fusarium*-infected maize well before the biochemical effects were clearly evident (Mirocha, 1983). In subsequent research, more sophisticated chronic feeding studies with swine have shown decreased skin temperature, depressed feed intake, reduced thyroid size, altered stomach conditions, increased albumin levels, and decreased $\alpha$-globulin levels after consumption of dry feed with as little as 1 - 3 mg·kg$^{-1}$ DON (Jacobsen et al., 1993; Rotter et al., 1994).

Although type B mycotoxins are among the least acutely toxic trichothecenes (e.g., DON, $LD_{50} = 46$ mg·kg$^{-1}$ mice, oral), chronic exposure is considered a significant risk for both humans and domestic animals (Canady et al., 2001). Most countries involved in the export and import of grain have imposed strict guidelines to regulate the trade of DON-contaminated cereals. In March, 2004, the Codex Committee on Food Additives and Contaminants reaffirmed the 2001 Joint FAO/WHO Expert Committee on Food Additives (JECFA) provisional maximum tolerable daily intake (PMTDI) for DON of 1 $\mu$g·kg$^{-1}$·day$^{-1}$ for cereals for use within human food or food processing (http://ec.europa.eu/food/fs/ifsieupositions/ccfac/archives/ccfac_04366_item14g_en.pdf). This regulatory limit for DON, and closely-related mycotoxins, will undoubtedly continue to evolve as more accurate risk-assessment models are developed based on more precise toxicological end-points (Miller, 2008).

### 2.4.2 Mechanism of Virulence for FHB

DON is regarded as a principal component of the necrotrophic phase of *Fusarium* pathogenesis of plant tissue (Atanasoff et al., 1994; Desjardins et al., 1996; Desjardins et al., 2000; Desjardins, 2006; Harris et al., 1999a; McCormick, 2003; Mesterházy, 2002;
Miller, 1994; Miller et al., 1985; Mirocha et al., 1997; Mudge, et al., 2006; Proctor et al., 1995; 1997; Snijders and Perkowski, 1990; Wang and Miller, 1987; 1988; etc.). Aside from inhibition of protein synthesis, as previously discussed, DON can induce a range of phytotoxic symptoms including light-dependent bleaching of detached plant tissues based on loss of chlorophyll, carotenoids (Bushnell et al., 2002; Seeland and Bushnell, 2001), and electrolytes within exposed plant-cell tissues (Cossette and Miller, 1995).

Electron microscopy and immunogold labeling techniques provide anecdotal evidence of DON, 15-AcDON and 3-AcDON toxicity in Fusarium pathogenicity (Kang and Buchenauer, 1999). Toxin diffusion into and within susceptible plant tissue occurs well in advance of Fusarium hyphal invasion (Figure 2.9). Without exception, toxin accumulation was well correlated with symptoms of Fusarium-related plant cell damage.

![Image](image.png)

**Figure 2.9** Immunogold localization of DON, 3-AcDON, and 15-AcDON within wheat spikelet (cv. Agent) infected with *F. culmorum*. Toxins detected within: **A**, Plant host cell wall (HCW) and ahead (see arrow) of hyphal fungal cell (FC) 1.5 days after inoculation (DAI). **B**, HCW of plant cells within the rachis 6 DAI. **C**, Parenchyma cells (PC) within a young kernel located three spikelets above inoculated spikelets 10 DAI. Secondary thickening (ST) noted within PC (Bars = 0.5 μm (Kang and Buchenauer, 1999).
Analytical methods to measure DON concentrations have also provided valuable input in terms of quantifying DON within developing cereal tissue. For example, Sinha and Savard (1997) used a competitive direct ELISA technique to measure DON concentrations within developing wheat heads and found that the rachis had the highest DON concentrations (93 ppm), followed by floral chaff (50 ppm), kernels (25 ppm), and peduncles (16 ppm). In a follow-up study, DON was detected below the site of injection into single florets at concentrations of hundreds of ppm, i.e. it was found within the kernels and rachis (Savard et al., 2000). The results of these studies are complemented by fungal colonization experiments based upon quantitative PCR assays of plant tissue before and after Fusarium pathogenesis. Nicholson et al. (1998) used quantitative PCR based on oligonucleotides from known \( F. \) graminearum DNA sequences to demonstrate greater fungal colonization of plant tissue by trichothecene-producing than non-producing mutant strains.

2.5 Transgenic Approaches to Limit Trichothecene Toxicity

The study and subsequent application of innate and transgenic mechanisms to instill enhanced “Type III” resistance to DON and structurally-similar trichothecenes within higher order plants is a complex and difficult task. Many variables (both known and unknown) affect host plant responses to Fusarium pathogenesis (Desjardins, 2006; Foroud and Eudes, 2009; Muehlbauer and Bushnell, 2003). For this reason, novel trichothecene-specific mechanisms are often initially evaluated within yeast and/or Arabidopsis as model eukaryotic test systems prior to evaluation within higher order plants. Such model eukaryotic test systems offer increased efficiency in terms of overall
convenience and decreased cost with minimal complexity, variability, and time to initial evaluation (Mitterbauer and Adam, 2002; Urban et al., 2002).

Four general mechanisms have been proposed as transgenic approaches to limit trichothecene, particularly DON, cytotoxicity within plants and eukaryotic test systems; 1) inhibition of trichothecene biosynthesis, 2) alteration of the target ribosomal site, 3) metabolic trichothecene detoxification, and 4) inhibition of trichothecene biosynthesis (McCormick, 2003; Mitterbauer and Adam, 2002). A fifth mechanism attributed to plantibodies and mimotopes as been added to this literature review based on novel applications of recombinant antibody engineering technology to limit mycotoxin-specific cytotoxicity.

2.5.1 Inhibition of Trichothecene Biosynthesis

Biosynthesis of trichothecenes within \textit{in vitro} test systems can be blocked by the addition of flavonoids, furanocoumarins, and various other naturally-occurring soluble phenolic compounds derived from plants (Bakan et al., 2003; Desjardins et al., 1988). For example, xanthotoxin and various other furanocoumarins, which are produced by a variety of plants, have been shown to be effective inhibitors of trichothecene biosynthesis at the preliminary step of trichodiene oxygenation. Unfortunately, the efficacy of such compounds is often limited as many fungi are capable of expressing enzymes that detoxify and limit the effectiveness of trichothecene biosynthesis inhibiting compounds (Spencer et al., 1990).
2.5.2 Alteration of Target Protein

Given that trichothecene mycotoxins inhibit protein synthesis by binding to RPL3, a possible mechanism to attenuate trichothecene cytotoxicity would be to develop ribosomal genes whose products do not bind trichothecene toxins. Some of the earliest work in this area was reported by Hobden and Cundliffe (1980) who reported ribosomal resistance to trichothecene produced by *Myrothecium verrucaria*. In fact, this work coupled with related research, initially confirmed that the RPL3 protein was the primary target of trichotheccenes (Fernandez-Lobato et al., 1990; Fried and Warner, 1981).

Harris et al. (1999b) introduced an altered RPL3 protein from rice (*Oryza sativa*) into tobacco (*Nicotiana tabacum*) cells to instill enhanced mycotoxin resistance. Expression of a rice cDNA encoded with a modified RPL3 based on a change from tryptophan to cysteine at residue 258 provided enhanced trichothecene resistance within tobacco (Harris and Gleddie, 2000; 2001, US Patent 606046). A similar approach was reported by Adam et al. (2000) who engineered a mutant tomato *Rpl3* gene to provide resistance to DON in yeast. Unfortunately, results based on modified plant *Rpl3* cDNA expression within transgenic tobacco plants were less promising as constitutive mycotoxin resistance was not achieved. Limited efficacy was attributed to a post-transcriptional effect which led to a preferential utilization of endogenous RPL3 protein as well as limitations associated with stoichiometry of RPL3 protein expression relative to rapidly elevated mycotoxin levels within infected tissue (Mitterbauer et al., 2004).
2.5.3 Metabolic Trichotheccene Detoxification

_In planta_ detoxification of DON, and structurally similar trichotheccenes, based on expression of trichotheccene detoxification and resistance genes is a rapidly-emerging and important field of study with many practical commercial applications. Comprehensive reviews of enhanced metabolic resistance to _Fusarium_ mycotoxins within plants is described in additional detail by Berthiller et al. (2007) and Boutigny et al. (2008).

_In planta_ expression of metabolic detoxification genes such as _Tri101_ is a common approach to instill enhanced trichotheccene tolerance. _Tri101_ is a gene located outside the principal trichotheccene biosynthetic gene cluster that encodes trichotheccene C-3 acetyltransferase, an enzyme which adds an acetyl ester group to the C-3 hydroxyl group on trichotheccenes (outlined in Section 2.3.2). Constitutive expression of _Tri101_ within plants and other model eukaryotic organisms enables a metabolic detoxification of DON to 3-AcDON, a compound with reduced eukaryotic cytotoxicity (Kimura et al., 1998a; 1998b). _Tri101_ has been expressed in a range of plants with promising results to show decreased trichotheccene cytotoxicity and prevention of FHB yield loss (reviewed in: Alexander, 2008; Kimura et al., 2006). Unfortunately, expression of _Tri101_ alone may not provide complete resistance as both _Fusarium_ and plants commonly produce deacetylation enzymes that can convert 3-AcDON back to DON. In fact, Mitterbauer and Adam (2002) stated that the efficacy of _Tri101_ expression within plants could be limited by a ‘futile cycling’ whereby plant carboxylesterases, as well as fungal enzymes such as _Tri8_ (McCormick and Alexander, 2002), could play a significant part in the metabolic
conversion of acetylated compounds back to more toxic C-3 hydroxyl precursors (e.g., 3-AcDON to DON).

The identification of trichothecene detoxification genes is not solely limited to those isolated from *Fusarium* species. Enzymes capable of trichothecene metabolism have also been isolated from other organisms. For example, potato tissue can rapidly metabolize, and limit the cytotoxicity of the trichothecene diacetoxyscirpenol based on an initial removal of the acetyl groups followed by cleavage of the trichothecene skeleton (Desjardins et al., 1992). In addition, constitutive over-expression of a UDP glycosyltransferases (UGTs) isolated from *Arabidopsis thaliana* can also decrease plant sensitivity to DON and 15-AcDON. Expression of UGTs enables the conjugation of sugars to trichothecenes thereby forming less toxic DON-3O-glucoside and 15-AcDON-3O-glucoside conjugates (Poppenberger et al., 2003). This work is supported by the earlier work of Miller and Arnison (1986), which showed that FHB-resistant wheat cultivars (e.g., ‘Frontana’) metabolized DON to a less toxic compound based on a biochemical conversion to at least three metabolites, one of which was a DON-glycoside conjugate.

Another means of enhanced, and perhaps more complete, metabolic resistance may involve the expression of bacterial genes responsible for the cleavage of the trichothecene epoxide group at C-12,13 (Figure 2.7). Although such de-epoxidation activity has been successfully demonstrated in bacteria (Fuchs et al., 2002), application of this technology within plants to consistently yield favorable results remains an area of future research.
2.5.4 Reduction of Intracellular Concentrations

The identification and application of candidate genes that encode for molecular efflux pumps may also be used to limit trichothecene cytotoxicity within plant cells. For example, Adam and Lemmens (1996) demonstrated enhanced trichothecene resistance based on *in planta* expression of the ABC (ATP Binding Cassette) transporter protein encoded by a Pleiotropic Drug Resistance gene (i.e. *Pdr5*) isolated from *S. cerevisiae* (Balzi et al., 1994). *Pdr5* is one of nine members of the (NBF-TMS$_6$)$_2$ subclass of ABC encoding genes present within the yeast genome. ABC transporter proteins are located in the plasma membrane of host cells and remove toxic substances using the energy of ATP hydrolysis (Decottignies and Goffeau, 1997; McCormick et al., 1999).

Expression of *Pdr5* genes is a promising strategy to complement other approaches. Muhitch et al. (2000) successfully demonstrated that co-expression of the *Fusarium* acetylation enzyme (TRI101), and *Saccharomyces* transporter protein (PDR5) resulted in increased resistance to the trichothecene 4,15-diacetoxysciprenol during germination and plantlet development of transgenic tobacco.

2.5.5 Plantibodies and Mimotopes

The expression of functional full-size antibodies and antibody fragments within plants has received extensive research interest within the last 15 years. Plant-produced antibodies, or “plantibodies” have been evaluated and applied mainly for production of biopharmaceuticals and immunodiagnostic agents as well as biocontrol agents to improve resistance against plant pathogens and cytotoxic herbicides (reviewed in Section 3.6.2.3).
Various antibodies with specificity to a wide range of mycotoxins are commonly used within standard immunoassay formats (e.g., Enzyme-Linked Immunosorbent Assays) to assess mycotoxin levels within contaminated grain (reviewed in Mirocha et al., 2003). However, despite reports of favorable efficacy, very few have been used as plantibodies to instill enhanced in planta resistance against Fusarium pathogenesis (Peschen et al, 2004). Of those tested, most are Fusarium-specific plantibodies expressed as fusion proteins to antifungal peptides (reviewed in Hu et al., 2008). In these cases, the Fusarium-specific antibody targets the pathogen while the fusion protein destroys it.

Surprisingly, only a very limited number of recombinant plantibodies have been applied to limit, or immuno-modulate the cytotoxic effects of low molecular weight mycotoxins. Yuan et al. (2000) reported the first in planta expression of an anti-mycotoxin gene. The functional, zearalenone-specific scFv fragment produced within the cytoplasm of transgenic Arabidopsis-leaf tissue was shown to have a similar binding affinity to a bacterially-produced scFv and its parent monoclonal Ab. Likewise, Yuan et al. (1999) were the first to report expression of two functional anti-idiotype peptides, or mimotope peptides for a low molecular weight mycotoxin (DON). Both mimotopes were antagonistic to DON-induced protein synthesis inhibition and cellular damage within a model eukaryotic test system.

These preliminary studies provide insights into the vast potential that antibody engineering can provide to design and express new and novel peptides that reduce (or perhaps eliminate) mycotoxin-specific cytotoxicity. The next literature review chapter provides a comprehensive overview of antibody engineering as applied to agricultural
biotechnology. Subsequent research chapters describe experiments at the heart of this Ph.D. project which were designed to develop, isolate and assess the efficacy of a unique single-domain antibody fragment with specificity for the mycotoxin 15-AcDON.
3 LITERATURE REVIEW 2: ANTIBODY ENGINEERING IN AGRICULTURAL BIOTECHNOLOGY

3.1 Introduction

Antibodies (Abs) are gamma globulin proteins produced by the immune system of vertebrates to recognize and bind to a virtually limitless repertoire of antigens (Ags). Abs are inherently unique, as they possess high affinity and binding specificity for a target Ag. These properties have made Abs invaluable reagents within various biotechnological and biomedical applications ranging from standard diagnostic immunoassays (e.g., ELISA) to integrated immunoaffinity-based systems designed to isolate, enrich, and purify specific target reagents (e.g., proteins, immuno-conjugates, etc.).

Initial efforts to produce in vitro Ag-specific Abs led to the introduction of hybridoma technology in 1975 in which Ag-stimulated B-cells were fused with their myloma counterparts to generate stable hybrid or “hybridoma” cell lines to enable large scale production of Ag-specific monoclonal Abs (mAb) (Köhler and Milstein, 1975). Subsequent advances in the fields of genetics, molecular biology and immunology have greatly enhanced applications of novel technologies required for the development, isolation, and application of recombinant Ab (rAb) fragments for an expanded range of applications. For example, routine use of phage display technology to screen combinatorial rAb libraries (reviewed in Winter et al., 1994; Hoogenboom, 2005) coupled with new software tools to support an expanding structure-function database (e.g., Harding et al., 2004) has opened unlimited opportunities to develop tailor-made rAb fragments. Moreover, on-going improvements associated with rAb optimization and subsequent expression in recombinant hosts offers the possibility of large-scale and low-
cost production platforms as an alternative to more expensive, laborious and often cumbersome methods associated with the mAb development using mammalian cell lines.

Antibody engineering has emerged as a new discipline focused on the identification, cloning, optimization and expression of Ag-specific rAb genes within various prokaryotic and eukaryotic host systems (Filpula, 2007; Holliger and Hudson, 2005; Jain et al., 2007; Lo, 2004; Maynard and Georgiou, 2000). This review presents an overview of established and emerging technologies available for the de novo generation of Ag-specific, rAb fragments as an alternative to traditional immunoassays based on monoclonal antibodies or polyclonal serum. Applications and future research needs specific to agricultural biotechnology and genetically-modified plants are presented as concluding remarks.

Key Words
Antibody; antigen; immunoassay; immunoaffinity; ELISA; recombinant antibody (rAb); antibody engineering; rAb fragment; phage display; combinatorial libraries; rAb optimization; rAb expression; Ab engineering.

3.2 Recombinant Antibody Fragments

The production of Ag-specific rAb fragments derived from full-size conventional Abs was first reported in the late 1980’s following the discovery of the genetic mechanism associated with Ab gene rearrangements. Concurrent advances in subcloning and expression systems coupled with the introduction of polymerase chain reaction (PCR) technology further enabled the recombinant expression of rAb fragments within bacterial hosts (initial reviews in Plückthun and Skerra, 1989; Winter and Milstein, 1991). In
general terms, the production of rAb fragments through these new processes involved the isolation of mRNA for specific Ab genes derived from either hybridoma, spleen cells, or lymph node leukocytes followed by reverse transcription PCR (RT-PCR) to form complementary DNA (cDNA) and eventual PCR amplification by using gene-specific primer sets to generate a complete Ab gene sequence (Maynard and Georgiou, 2000).

Given the inherent reliance on PCR amplification, the most significant initial advancements were related to the application of optimized oligonucleotides or so-called primers to anneal and amplify target rAb genes. Within ten years of adoption of PCR, numerous primer sets were published based on N-terminal sequences of isolated rAb genes (Benhar and Pastan, 1994), Ab leader sequences (Larrick et al., 1989), and known variable domain framework sequences.

A wide variety of rAb fragments have been produced, characterized, and applied in various biological systems (reviewed in Hudson, 1998; 1999; Little et al., 2000; Holliger and Hudson, 2005; Yau et al., 2003a). The following section provides a general description of the three most common and well documented rAb fragments with potential applications in agricultural biotechnology.

### 3.2.1 Fab Fragment

The term ‘Fab’, or fragment for antigen binding (Better et al., 1988), refers to a relatively large (MW ≈ 50 – 55 kDa) heterodimer molecule comprised of two polypeptide chains, one containing the constant and variable light chain domains (C\textsubscript{L} + V\textsubscript{L}) of a parent Ab paired with its corresponding variable and first constant regions (C\textsubscript{H1} + V\textsubscript{H}) (Jeffrey et al., 1993) (Figure 3.1).
Figure 3.1  Schematic representation (left) and ribbon diagram (right) of recombinant antibody fragment (Fab). Constant and variable light chain domains (C_L + V_L) and corresponding first constant and variable heavy chain domains (C_H1 + V_H), and target Ag (i.e., Digoxin) are labeled accordingly (adapted from Joosten et al., 2003 and Jeffrey et al., 1993).

Fabs have the advantage of enhanced durability within in situ applications, as they are inherently more stable and less prone to dissociation and proteolytic degradation relative to other conventional rAb fragment formats (e.g., scFv). Enhanced stability is attributed to the presence of a covalent disulfide bond which serves to retain tertiary folding and to facilitate more extensive interface and non-covalent interactions between the two paired immunoglobulin chains or four Ab domains (Maynard and Georgiou, 2000). Despite their relatively large size relative to other rAb fragment types, Fab proteins are amendable to standard Ab engineering techniques to improve attributes associated with biological function, recombinant expression, affinity for target Ag, etc. (reviewed by: Filpula, 2007; Holliger and Hudson, 2005; Maynard and Georgiou, 2000).

3.2.2 scFv Fragment

The term single-chain variable fragment (scFv) refers to a rAb fragment in which the variable heavy and light domains (V_H and V_L, respectively) of a parent immunoglobulin (reviewed in Chapter 1) are joined by a hydrophilic and flexible peptide
linker (Figure 3.2) (Bird et al., 1989; Huston et al., 1988). The order of the domains can be designed for expression as either V\textsubscript{H}-linker-V\textsubscript{L} or V\textsubscript{L}-linker-V\textsubscript{H} (Huston et al., 1991; 1995). In most cases, the length of the peptide linker is 15 amino acids based on a (Gly\textsubscript{4}Ser\textsubscript{3}) configuration (reviewed in Huston et al., 1995).

![Diagram of recombinant single chain variable fragment (scFv) antibody](Image)

**Figure 3.2** Schematic representation (left) and ribbon diagram (right) of recombinant single chain variable fragment (scFv) antibody. Variable light and heavy chain domains (V\textsubscript{L} and V\textsubscript{H}, respectively) and synthetic peptide linker are labeled accordingly (adapted from Joosten et al., 2003 and Filpula, 2007).

Specific length and composition of the peptide linker can have a dramatic impact on post-translational folding and valency of scFv fragments (Robinson and Sauer, 1998; Turner et al., 1997). For example, Atwell et al., (1999) demonstrated that three amino acid linkers often form scFv dimers whereas linkers with two residues or less induce the formation of trimers. In general, increasing the length of linker will lead to monomeric variants. However, monomeric and multimeric scFv variants have been reported with linkers of up to 30 residues (Desplancq et al., 1994; Nieba et al., 1997).
Once valency is accounted for, one cannot assume that all scFv fragments possess comparable Ag-binding affinity to larger Ab formats (e.g., mAb and Fab) because they all have the same V_H / V_L domain pairing. Numerous publications, based on various Ags, have demonstrated that relative scFv affinity is best described on case by case basis. For example, Choi et al. (2004) noted that a scFv fragment raised against deoxynivalenol (haptenic Ag, MW ≈ 296 Da) had a binding affinity that was approximately two orders of magnitude less than the parent mAb. However, based on a similar comparison for the same Ag, Wang et al. (2007) noted no differences between mAb and scFv binding affinity.

Similar variability has been noted for scFv stability and efficiency of expression. In general terms, post-translational scFv folding and function is greatly influenced by specific expression systems and growth conditions (i.e., choice of expression vector and recombinant host, etc.) as well as method of purification. In addition, factors such as proteolytic degradation of the linker sequence (Whitlow et al., 1993) and formation of insoluble inclusion bodies (Choi et al., 2004) have great influence on scFv stability and expression.

Various techniques have been used to overcome such limitations. For example, Reiter et al. (1994) reported on the formation of disulfide-stabilized Fvs (i.e., dsFvs) through the introduction of cysteine residues between the V_L and V_H domains which resulted in the formation of an inter-chain disulfide bond which thereby conferred greater rAb stability. As well, molecular chaperones have been used as part of scFv expression
strategy to assist in post translational scFv folding and to increase efficiency of expression (reviewed in Arbabi-Ghahroudi et al., 2005).

3.2.3 \( V_{H}H \) Fragment

The term “\( V_{H}H \)” refers to the variable domain of camelidae family heavy chain Abs (HCAbs). \( V_{H}H \) fragments are among the smallest intact Ag-binding fragments known (\( \approx \) average 14 to 15 kDa, 118 - 136 amino acid residues) (Figure 3.3).

\[ \text{Figure 3.3} \quad \text{Schematic representation (left) and ribbon diagram (right) of recombinant HCAb variable domain (\( V_{H}H \)) of antibody “AMD9”. CDR 1, 2, and 3 in ribbon diagram are labeled accordingly (adapted from Joosten et al., 2003 and Desmyter et al., 2002).} \]

The hallmark of \( V_{H}H \) domain resides in key hydrophobic to hydrophilic amino acid substitutions at former \( V_{L} \) interface which imparts increased solubility and stability (e.g., resistance to aggregation). Despite the absence of \( V_{L} \) and \( V_{H} \) combinatorial diversity, single domain \( V_{H}H \) fragments have demonstrated high affinity binding to a wide range of Ag types (Ghahroudi et al. 1997; Muyldermans, 2001). HCAbs are believed to compensate for the loss of \( V_{L} \) domain through extended CDR3 loop regions and a higher rate of somatic hypermutations to form a \( V_{H}H \) paratope that can form a large antigen-
binding repertoire and additionally can penetrate into and conform to clefts and cavities of Ag epitopes (reviewed in Muyldermans, 2001). An example of this unique ability is V_{H}H binding to active sites of enzymes that are not typically recognized by conventional rAb fragments (e.g., Desmyter et al., 1996; 2002). Numerous reviews are available which document Ag binding characteristics and potential applications of V_{H}H fragments (e.g., Holliger and Hudson, 2005; Joosten et al., 2003; Muyldermans, 2001).

Single domain V_{H}H fragments offer a number of advantages relative to larger and more complex rAb fragments such as scFv or Fab. DNA encoding the variable domain of HCAb can be readily isolated from peripheral leukocytes and subcloned into various phage display vectors for highly efficient V_{H}H library generation since no synthetic linker is required for the pairing of V_{H}-V_{L} domains (Ghahroudi et al., 1997). Another beneficial characteristic of V_{H}H is its hydrophilic amino acid substitutions within FRs (Muyldermans, 2001). Consequently, expression in hosts such as *E. coli* tends to produce high yields of soluble, stable V_{H}H protein that is inherently free of problems associated with protein aggregation and proteolytic degradation common to scFv expression (Arbabi-Ghahroudi et al., 2005). Finally, recent experiments have demonstrated that V_{H}H fragments possess enhanced functionality and stability over a range of adverse temperature and solvent regimes where current immunoassays do not typically function (e.g., Goldman et al., 2006; Ladenson et al., 2006).

The understanding of amino acid sequences of V_{H}H fragments provides interesting and useful techniques to improve expression of non-dromedary Abs. For example, it is possible to replace hydrophobic residues on the V_{H} side of a conventional rAb fragment
with ‘V_{H}H-like’ residues to minimize limitations associated with insolubility and non-specific binding. This process is known as ‘camelization’ and can be used with V_{H} fragments derived from various animal sources (Davies and Riechmann, 1994; Davies and Riechmann, 1996a; Tanha et al., 2001).

### 3.3 Development and Types of Antibody Libraries

The concept of Ab libraries was established following the elucidation of the genetic mechanisms associated with the creation of immunoglobulin gene diversity (Tonogawa, 1983) and the advancement of technologies associated with PCR for amplification of the variable domains of immunoglobulin genes (Orlandi et al., 1989; Songsivilai et al., 1990). In principle, rAb libraries may be classified as immunized, naïve, semi-synthetic, or synthetic according to the genetic material used for library construction.

#### 3.3.1 Immunized Libraries

Immunized rAb libraries are constructed from DNA isolated from peripheral leukocytes of a host that has been repeatedly exposed to a single target immunogen or group of immunogens. Library construction occurs through a stepwise process whereby the B cell genes encoding Ab variable domain regions are amplified by PCR and subcloned into vectors to enable expression of different rAb fragment formats including V_{H}H (Ghahroudi et al., 1997; Goldman et al., 2006; Ladenson et al., 2006), scFv (Clackson et al., 1991; McCafferty et al., 1990) and Fab (Huse et al., 1989; Orum et al., 1993; Persson et al., 1991).

The inherent advantage of an immunized library format resides in the fact that peripheral leukocytes, and therefore high concentration of corresponding mRNA used for
library construction, are the product of the process of affinity maturation (Hoogenboom et al., 1998). Although the development of a rAb library based on an immunogen-specific response improves the probability of isolating Ag-specific rAbs, overall library size and diversity is often limited relative to other non-biased library formats (Burton et al., 1991; Clackson et al., 1991).

Depending on the nature of the Ag and overall test system, the development of hyper-immunized libraries can present a number of technical challenges and potential drawbacks associated with isolating rAbs against self-Ags or against Ags that are extremely toxic or chemically unstable. Immunological tolerance is another factor which can reduce the diversity of natural in vivo repertoire. In addition, one must account for the various logistical, financial and ethical considerations associated with the immunization protocol to ensure a robust and predictable response to the Ag of interest (Willats, 2002; Hoogenboom, 1997).

3.3.2 Naïve Libraries

Naïve libraries are prepared according to the same methodology used for construction of immune libraries except that the animal has not been hyperimmunized with the Ag of interest. The major source of immunoglobulin genes are peripheral blood lymphocytes (PBLs), bone marrow, tonsils, and spleen cells. IgM is the preferred immunoglobulin isotype as it is more diverse and has not been subjected to bias or previous Ag selection (Dörsam et al., 1997; Griffiths et al., 1993; Little et al., 1993; Marks et al., 1991; Schier et al., 1995).
Library size plays a vital role in successfully isolating Ag-specific rAb fragments. This concept has been proven in several reports in which naïve libraries of medium size have only resulted in the isolation of Abs with micromolar affinities (Marks, et al., 1991; Griffiths et al., 1994). Accordingly, a library size of approximately $10^{10}$ is likely to yield Abs with affinities in the low nanomolar range (Vaughan et al., 1996). These results demonstrate that size and heterogeneity of such libraries have critical roles in isolating high affinity Abs without prior immunization. An advantage of naïve libraries, in particular larger-sized ones, is that they are often not antigenically pre-disposed to self-antigens. As such, large, diverse naïve libraries are a fit as a universal source of Ab binders (Vaughan et al., 1996).

3.3.3 Synthetic / Semi-Synthetic Libraries

Methodologies associated with synthetic libraries combine the advantages of naïve libraries (e.g., structural diversity, etc.) with on-going advancements associated with improved knowledge of rAb structure and function. Accordingly, these technologies enable in vitro production of high affinity rAbs with application against a range of target Ags that are not affected by inherent controls which may limit expression or bias an immunogenic response to a target Ag (Fellouse et al., 2007; Knappik et al., 2000; Winter and Milstein, 1991).

One of the first semi-synthetic libraries was generated by using a repertoire of $V_H$ genes, combined with a synthetic CDR3 construct, and displayed on phage surface as either scFv (Hoogenboom et al., 1992) or Fab (Barbas et al., 1992) fragments. The complexity and diversity was further expanded using a repertoire of $V_L$ genes in
conjunction with initial library formats (de Kruif et al., 1995; Nissim et al., 1994). Second generation libraries evolved from the development of synthetic Ab scaffolds that express well in *E. coli* to help overcome limitations associated with post-translational rAb folding (Knappik and Plückthun, 1995). It is also possible to construct fully synthetic rAb libraries using available DNA sequences of human immunoglobulins (Andris et al., 1995; Cook and Tomlinson, 1995; Tomlinson et al., 1992; Winter, 1998) followed by total gene synthesis for expression in *E. coli* based on optimized rAb scaffolds (Griffiths et al., 1994; Knappik et al., 2000; Krebs et al., 2001).

### 3.4 Display Systems

High throughput screening of rAb libraries is made possible through techniques that establish a direct physico-chemical link between a gene (i.e., genotype) and the resultant rAb fragment (i.e., phenotype). This linkage allows isolation of an Ab fragment with desired Ag binding properties from a large, pooled rAb repertoire, while the genetic material is available for further isolation and engineering. rAb fragments are most commonly displayed on phage, bacteria, yeast, and ribosomes (Li, 2000; Yau et al., 2003a).

#### 3.4.1 Phage Display

Phage display was first demonstrated by Smith (1985) who reported expression of foreign peptides as a fusion with pIII viral coat protein on the surface of non-lytic filamentous phage. Within five years of Smith’s demonstration of a direct link between phenotype, i.e., as protein expressed on the phage surface, to genotype, i.e., as foreign DNA within phage genome, McCafferty et al. (1990) used phage display techniques to
isolate Ag-specific scFv fragments from a diverse filamentous phage-scFv display repertoire. Since the time of these reports, phage display technology has matured into a widely adopted technique for selection of rAb fragments from very large phage-display libraries.

Phage display techniques are now commonly used to produce Ab fragments with optimized binding affinity, stability, activity (reviewed by Forrer et al., 1999) and to isolate Abs which were previously considered to be very difficult to obtain through conventional methods (Griffiths et al., 1993; Hoogenboom and Chames, 2000). Numerous reviews highlight the diverse applications of phage-display technologies (e.g., Hoogenboom et al., 1998; Paschke, 2006; Smith and Petrenko, 1997; Willats, 2002; etc.).

3.4.1.1 Filamentous Bacteriophage

Filamentous bacteriophage (Ff) are viruses (e.g., F1, M13, fd, etc.) that can infect and replicate within gram-negative bacteria (e.g., E. coli) without killing the host cell (Russel et al., 2004). These viruses are ideal for applications within rAb engineering since the Ff genome is inherently small and tolerates insertions into non-essential regions without loss of bacterial infectivity. During phage assembly, fusion proteins are expressed on a particular viral coat protein while genetic information encoding the displayed protein is packaged within the single-stranded DNA (ssDNA) of nascent phage particles (Smith and Pertenko, 1997). Direct genotype to phenotype coupling ensures that phage produced within the same infected bacterial cell are comprised of identical clones (Paschke, 2006).
The defining characteristic of Ff structure is a circular 6.4 kbp ssDNA genome encased by a long, flexible tube comprised of ≈ 2,700 copies major coat protein (pVIII), with a minor coat proteins (pIII, pVI, pVII, and pIX) expressed at phage tips (Figure 3.4) (Yau et al., 2003a). Of these coat proteins, pIII is the most extensively used and well characterized phage-display format since it is more amendable than the other coat proteins to large protein insertions without loss of phage infectivity (reviewed in Hoogenboom et al., 1998; Hoogenboom and Chames, 2000; Russel et al., 2004; Smith and Petrenko, 1997; etc.).

3.4.1.2 Phage vs. Phagemid Vectors

Phage display rAb libraries are constructed by cloning a diverse PCR amplified Ab repertoire into either a phage or phagemid vector (Hoogenboom et al., 1992; Winter et al., 1994). Regardless of library type (i.e., phage or phagemid), the salient goal is to construct a heterogeneous and robust mixture of phage clones which collectively display rAb–phage fusions capable of target or “Ag-specific” recognition and replication to produce identical progeny phage in large scale (Smith and Petrenko, 1997).
Both phage and phagemid vectors are designed so that the target rAb gene is inserted between a signal sequence and the coat protein gene (e.g., pIII). Likewise, both types of vectors carry antibiotic resistance selectable markers, as well as a phage-derived origin of replication to enables packaging of foreign DNA into viral ssDNA and production of nascent phage progeny which display rAb fused to surface phage coat protein (Figure 3.5) (Russel et al., 2004; Smith and Pertenko, 1997).

Phage vectors are derived directly from the Ff genome whereby the recombinant protein is cloned as a fusion to phage coat protein (e.g., pIII) for production of multivalent rAb phage protein progeny. Conversely, a phagemid vector is a plasmid-based sequence harbouring gene III and intergenic region of Fd phage fusion gene under control of a weak to moderate bacterial promoter such as LacZ. Phagemid vectors also have a second
origin of replication to allow propagation as a plasmid within host *E. coli* (Figure 3.5).

Many phagemid vectors also have an amber stop codon inserted between end of desired protein and pIII gene to enable soluble rAb expression within non-suppressor strains of *E. coli* (e.g., HB2151) (Russel et al., 2004; Smith and Pertenko, 1997).

![Diagram](image)

**Figure 3.5** Phage (left) and phagemid (right) vectors to construct rAb phage display libraries through the minor coat protein pIII. Ff ori = filamentous phage origin of replication. Plasmid ori = plasmid origin of replication. TAG = Amber stop codon. Sig. = filamentous phage leader sequence (adapted from Russel et al., 2004).

Phage production using phagemid vectors is only possible when additional phage proteins are provided by helper phages through a process known as “phage-rescue” (reviewed within Paschke, 2006, etc.) (Figure 3.5). Use of helper phage is, therefore,
very critical as improves the relative proportion of univalent hybrid phage progeny carrying rAb-phagemid genome to wild-type or “bald” phage based only on phage genome (Russel et al., 2004). Helper phage can also impact resultant phage valency within pIII phagemid systems to promote production of monovalent phages carrying only one rAb fragment pIII fusion protein per phage particle (Paschke, 2006; Russel et al., 2004).

Finally, regardless of vector type, during library construction, it is essential to maintain stringent control of fusion protein expression using controllable bacterial promoter such as LacZ (for example by using Glucose as repressor) through selectable markers and consistent techniques during all propagation steps as continuous expression of rAb-phage coat fusion proteins increases the metabolic burden to host E. coli cells. Such techniques are important to ensure consistent selection pressure to limit any ecological advantage for non-functional clones which limit library efficiency and function (Paschke, 2006).

3.4.1.3 Panning

The selection of Ag-specific Abs from a phage display library occurs through a procedure known as ‘bio-panning’ or simply ‘panning’. Although this process can involve many variations and modifications, it relies mainly on the stepwise isolation and amplification of phage displaying Ag-binding Ab fragments (reviewed in Maynard and Georgiou, 2000; Paschke, 2006; Smith and Pertrenko, 1997; etc.).

After exposure of the full phage-display rAb repertoire to the target Ag (e.g., coated onto a solid surface or present in solution or even on the cell surface), washing steps are
used to remove phage carrying non-specific Abs from those displaying Ag-specific Abs. The latter are desorbed through low or high pH elution prior to amplification in phage host (typically *E. coli*) (Figure 3.6). After each round of panning, amplified phage are subject to increased selection pressure and washing to continue phage enrichment and amplification based on Ag-binding properties of phage-displayed rAbs (Paschke, 2006).

![Phage display panning cycle](image)

**Figure 3.6** Phage display panning cycle: 1.) rAb-displaying phages are added to immunotubes coated with target Ag; 2.) Washing is performed to remove non-binding phages; 3.) Positive-binding phage are eluted; 4.) Eluted phage are used to infect *E. coli*; 5.) Positive clones are plated as *E. coli* plaques or colonies; 6.) Helper phage (if phagemid system) is added for phage rescue to amplify positive clones; 7.) Positive phage are purified for reintroduction into next round of panning (de Bruin et al., 1999).

High affinity phage display Abs are commonly selected within three to four rounds of panning (de Bruin et al., 1999). After panning, rAb fragments are typically expressed in *E. coli* and purified for subsequent characterization, and application.
3.4.2 Bacteria Display

Peptide display on the surface of bacteria was first described well over a decade ago (Georgiou, et al., 1993). Since the initial reports of recombinant peptide expression in prokaryotes, fully functional Ab fragments have been displayed on surface of *E. coli* (Francisco et al., 1993; Fuchs et al., 1991). As noted for phage display, bacterial display libraries can also be used to select rAbs with improved affinity for target Ag (Daugherty et al., 1998; 1999).

A limitation associated with the expression of foreign peptides on the surface of gram-negative bacteria (e.g., *E. coli*) is the physiological barrier associated with an extensive network of macromolecules within the bilayer cell envelope. However, this limitation can be circumvented: peptidoglycan chaperones have been used to facilitate transport and anchoring of recombinant Ab fragments through both the inner and outer membranes without causing an adverse effect on cell growth and viability (Fuchs et al., 1991). Another way to address this problem is to select an appropriate leader sequence and a surface-localized carrier protein (Yau et al., 2003a).

It is also possible to express rAbs on the surface of gram-positive bacteria. For example, scFv expression has been reported on the surface of *Staphylococcus xylosus* and *S. carnosus* (Gunneriusson et al., 1996) Ab display on gram-positive bacteria has been shown to provide efficient rAb secretion and post-translational folding.

3.4.3 Yeast Display

As in bacterial surface display, the phenotype and genotype linkage for yeast surface display models is comprised by the protein on the cell surface and the corresponding
DNA within the cell. Given this direct linkage, an additional subcloning step is not required after selection of an Ag-binding cell (Yau et al., 2003a).

Yeast display of rAbs offers many of the advantages of bacterial display. Yeast has a thick, rigid cell wall which enables stable maintenance of surface-displayed proteins (Georgiou et al., 1997). In contrast to typical prokaryotic expression systems (e.g., E. coli), yeast possess protein folding and secretory mechanisms of higher order eukaryotic (e.g., mammalian) systems. As such, yeast display alleviates some biases associated with prokaryotic protein expression and folding (Boder and Wittrup, 1997).

Despite these advantages, experience has shown that yeast-display Ab libraries tend to have limited transformation efficiency, leading to smaller and less diverse repertoires relative to phage or bacterial display (Yau et al., 2003a). Nevertheless, high affinity rAb fragments have been isolated from yeast display libraries. For example, Boder and Wittrup (1997) successfully used yeast-surface display to screen a diverse peptide library to select binders to a soluble fluorescein tracer. Likewise, Kieke et al., (1997) reported successful isolation of an anti-T cell receptor scFv using a yeast-surface display library.

3.4.4 Ribosome Display

Ribosome display represents an alternative to expression of Ab fragments on the surface of phage, yeast or bacterial cells. As in other display systems, ribosome display libraries are designed based on a direct link between peptide / Ab phenotype and genotype. Unlike other library formats, ribosome display is entirely in vitro or cell-free (reviewed in Hanes and Plückthun, 1997; Schaffitzel et al., 1999). Ribosome display is unique as the phenotype to genotype construct is designed to include a spacer sequence
with no stop codon such that, when the nascent Ab fragment is translated from mRNA, both the mRNA and the Ab remain bound to the ribosome molecule to form an ‘ARM’ (Antibody-Ribosome-mRNA) complex (Figure 3.7) (He and Taussig, 1997).

Figure 3.7 Ribosome display panning cycle. 1.) Double-stranded DNA (dsDNA) transcription to form messenger RNA (mRNA). 2.) In vitro translation of mRNA to form ARM (Antibody-Ribosome-mRNA) complexes. 3.) Exposure of ARM complexes to immobilized target Ag for affinity selection or Ag-specific panning. 4.) Elution and isolation of mRNA from Ag-specific ARM complex(es). 5.) Reverse transcription PCR amplification of target mRNA to form dsDNA for continued panning or transformation into host organism (e.g., E. coli) (adopted from Yau et al., 2003a).

A key advantage of cell-free systems is that very large libraries can be handled since transformation efficiency is not a limiting factor as it is for other systems. In addition, in vitro systems are not impacted by ongoing random mutation of foreign DNA imparted by...
the host organism which may limit library diversity. As an example, the typical size limit associated with transformation efficiency for bacterial display libraries ranges from $10^{10} – 10^{11}$ clones (Yau et al., 2003a). With ribosome display, however, libraries with up to $10^{15}$ members can be created and screened (Roberts, 1999).

Ribosome display also enables the incorporation of continuous diversification during each panning cycle using PCR-based mutagenesis. As a result, ribosome display is a good format to select Ab fragments with improved $K_D$ (dissociation constants) and robust Ag-binding affinities (Yau et al., 2003a). For example, Hanes et al. (1998) reported the isolation of scFvs from a ribosome-display library with affinities to target Ag in the picomolar range.

### 3.5 Optimization of Recombinant Antibodies

Ab fragments such as scFv, Fab, and $V_H$H often display lower affinity than their parental, full-size counterparts (Adams and Schier, 1999; Huston et al., 1996). A number of strategies can be used to overcome this limitation, thus enabling isolation of high-affinity Ag-specific rAb fragments. For example, panning procedures vs. target Ag can be optimized and *in vitro* mutagenesis (where the natural affinity maturation process is mimicked) can be performed (Adams and Schier, 1999).

#### 3.5.1 Optimized Panning Procedures

Although optimization of the selection or panning process (Figure 3.6) does not inherently improve rAb characteristics, it allows for more efficient recovery or selection of high-affinity Ag-specific rAb fragments from a diverse library. Panning strategies used to increase the probability of isolating high-affinity rAbs have generally consisted of
gradually decreasing the Ag concentration during successive rounds of panning (Strachan et al., 2002). In the case of panning against low molecular weight haptenic Ags, panning strategies often use soluble or free hapten to elute the rAb from target Ag. Likewise, structurally-similar hapten analogues can be preincubated with the phage-display rAbs prior to exposure to the target hapten conjugate. Finally, subtractive panning, where the rAbs are incubated with the carrier protein prior to incubation with the hapten, can also be used to improve the probability of selecting Ag-specific, high affinity rAbs (reviewed in Sheedy et al., 2007).

As more knowledge is gained on Ab structure through X-ray crystallography, specific mutations can be targeted to modify and improve rAb specificity and affinity for target Ag (Korpimäki et al., 2003; Lamminmäki et al., 1999). Ab engineering provides excellent tools to tailor the properties of rAb fragments with respect to affinity, specificity and performance for different applications (Valjakka et al., 2002).

3.5.2 In vitro Affinity Maturation

Because of the relatively low affinity of rAbs isolated from naïve libraries, the process of somatic hypermutation must be mimicked through antigen-driven affinity maturation (Irving et al., 1996). In vitro affinity maturation by site-directed or random mutagenesis is not limited by the restrictions inherent to the natural somatic hypermutation mechanisms (Parhami-Seren et al., 2002). Affinity maturation in vitro can, therefore, generate extended and functional variability in rAb molecules (Borrebaeck and Ohlin, 2002), and lead to selection of rAb fragments with increased affinity and specificity for target Ag (Roberts et al., 1987; Wu et al., 1998; 1999).
Strategies to affinity mature Abs and fragments thereof in vitro can be grouped into the following categories: random mutagenesis, site-directed mutagenesis, and shuffling. Random mutagenesis consists, as its name implies, of the introduction of mutations randomly throughout the gene. Random mutagenesis can be subgrouped into error-prone PCR and bacterial mutator strains. Site-directed mutagenesis includes mutational hot spots and parsimonious mutagenesis, as well as any mutational strategy which assigns the mutations to given positions or residues within an Ab’s wild-type gene. Shuffling encompasses chain shuffling, DNA shuffling and staggered extension process. The most frequently used strategies are those of site-directed mutagenesis, where mutations are directed to specific CDRs or framework regions (Irving et al., 1996), and error-prone PCR, where mutations are randomly introduced across the gene.

3.5.2.1 Random Mutagenesis

3.5.2.1.1 Error-Prone PCR

Error-prone PCR uses low-fidelity polymerization PCR conditions to introduce point mutations randomly over a gene sequence (Stemmer, 1994). The introduction of random mutations in vitro is an efficient method for increasing both specificity and affinity, as long as a strong selection pressure is applied during the panning stage (Miyazaki et al., 1999).

3.5.2.1.2 Bacterial Mutator Strains

Affinity maturation of rAbs and increase in their expression levels has been achieved by using E. coli mutator cells (Coia et al., 2001a; 2001b; Irving et al., 1996; 2002). This approach consists of the selection of rAbs from a library followed by gene
diversification or mutagenesis through amplification in a bacterial mutator strain of *E. coli* such as MutD5, a conditional mutant which produces single base substitutions (transversions or transitions) at high frequency compared to normal *E. coli* cells. Such mutator strains can produce a large number of mutant rAbs which can then be selected by phage-display or other methods (Irving et al., 1996). For example, the affinity of an scFv for the glycoprotein glycophorin was increased by passaging the rAb construct through such a bacterial mutator strain. A single point mutation resulted in a 10-fold increase in the scFv binding affinity, and another point mutation located in a framework region near the CDR3 of the V<sub>L</sub> resulted in a 1,000-fold increase in affinity (Irving et al., 1996). The effects of these point mutations illustrate the power of random mutagenesis in generating high-affinity mutants that could not be obtained by site-directed mutagenesis. However, four to ten rounds of mutation or passage through mutator cells are required to obtain high affinity mutants (Coia et al., 2001a).

### 3.5.2.2 Focused Mutagenesis

#### 3.5.2.2.1 Site-Directed Mutagenesis

Improvements in affinity can be achieved *in vitro* by site-directed mutagenesis, where specific or selected residues are mutated at predetermined locations within target rAb genes. Following mutagenesis, a library is constructed and screened for high-affinity mutants. For example, Davies and Riechmann (1996b) have investigated the effect of randomizing CDRs 1 and 2 residues in V<sub>H</sub> domains specific for both haptens and protein antigens. This research was successful in terms of generating randomized repertoires which were displayed on phage and affinity selected to improve Ag binding.
3.5.2.2.2 Mutational Hot Spots

Chowdhury and Pastan (1999) have developed a strategy that enables the isolation of rAb fragments with increased affinity from small phage-display libraries. Their approach is based on the fact that the DNA encoding the variable domains of Abs contains “mutational hot spots”, or nucleotide sequences naturally prone to hypermutations during the in vivo affinity maturation process. Several types of hot spots have been suggested, such as direct and inverted repeats, palindromes, secondary structures and certain consensus sequences (Chowdhury and Pastan, 1999). Several consensus hot spot sequences have been studied in great details; one of these sequences is the tetranucleotide A/G-G-C/T-A/T (Chowdhury and Pastan, 1999). The serine codons AGY, where Y can be either C or T, are other hot spot consensus sequences found in Ab genes that can be used as targets for mutagenesis.

Hot spot mutagenesis techniques have been used to affinity mature rAb fragments as a precursorary step to develop Ag-specific high affinity rAbs (e.g., Chowdhury and Pastan, 1999; Li et al., 2001). For example, hot spot mutagenesis was used to generate a scFv fragments with high affinity to mesothelin, a cancer protein Ag. The affinity of the mesothelin-specific scFv PE38 was found to be 11 nM (Chowdhury and Pastan, 1999). Improved affinity was desired to increase its cytotoxicity so that it could serve as a potent immunotoxin. DNA sequences present in the rAb variable domains (hot spots) were selected and random mutations were introduced in the light chain CDR3 (Chowdhury and Pastan, 1999). Thirty-two hot spots were identified in the scFv, 14 being located in the variable heavy domains, and 18 in the variable light domains. A library with the affinity-
matured clones was constructed and panning of the library yielded several mutants with a 15- to 50-fold increase in affinity. Mutagenesis of the same original library but outside of the hotspots resulted in a mutated library from which the highest-affinity clone selected only had a four-fold increased affinity compared to the wild-type Ab (Chowdhury and Pastan, 1999).

### 3.5.2.2.3 Parsimonious Mutagenesis

Parsimonious mutagenesis was developed by Balint and Larrick (1993) as a technique whereby all three CDRs of a variable region gene are simultaneously and thoroughly searched for improved variants in libraries of manageable size (Balint and Larrick, 1993; Schier et al., 1996a; 1996b; 1996c). In parsimonious mutagenesis, synthetic codons are used to mutate about 50% of all targeted amino acids while keeping the other 50% of targeted residues intact (wild type) (Chames et al., 1998). rAb libraries can be constructed with low-redundancy “doping” codons and biased nucleotide mixtures designed to maximize the abundance of combining sites with predetermined proportions of pre-selected sets of alternative amino acids (Balint and Larrick, 1993).

This technique has several advantages over standard mutagenesis procedures, one of which is the total number of substitutions per Ab gene is reduced thereby resulting in a larger number of well-folded and potentially active binders in the library (Chames et al., 1998). Random mutagenesis may target residues essential to Ag-binding, leading to a library of mutants that will be mostly non functional, whereas parsimonious mutagenesis will preserve these essential residues, resulting in a library of mainly functional clones (Chames et al., 1998).
3.5.3 Optimized Panning Procedures

Shuffling of rAb genes can be accomplished in several ways: chain shuffling, DNA shuffling and staggered-extension process (StEP), and through variations of these techniques. Chain shuffling consists in shuffling heavy and light chain variable regions of Abs. In DNA shuffling, the Ab DNA is digested with DNase I, and randomly reassembled and amplified by PCR. StEP is also a PCR-based method, where template switching, caused by shortened extension time, shuffles various portions of several parental Ab genes.

3.5.3.1 Chain Shuffling

Chain shuffling can generate high-affinity rAbs (e.g., scFv) from immunized animals quite rapidly (Kang et al., 1991). Since shuffling approaches mimic somatic hypermutation, they are believed to be more efficient than random or site-directed mutagenesis in producing functional rAbs (Ness et al., 1999; 2002). The heavy and light chains of rAbs isolated from an immune library can be recombined, thereby generating a vast number of functional rAbs from an initially limited genetic repertoire (Kang et al., 1991).

For chain shuffling to be an effective mutagenesis strategy, the pre-selection of Ag-binding rAbs from an immune library is required prior to invoking the shuffling process. In other words, chain shuffling is only feasible to improve rAb affinity within immunized libraries (Park et al., 2000). Accordingly, chain shuffling is not applicable to naïve rAb libraries since heavy and light chains available within the library have not been exposed to target Ag. Moreover, experiments have shown that individual chains might
discriminate among partners, rendering the shuffling process difficult between naïve and somatically mutated chains (Kang et al., 1991).

3.5.3.2 DNA Shuffling by Random Fragmentation and Reassembly

DNA shuffling (Stemmer, 1994; Stemmer et al., 1995) is based on repeated cycles of point mutagenesis, recombination and selection, which should allow in vitro molecular evolution of complex sequences such as proteins. It somewhat mimics the natural mechanisms of molecular evolution (Minshull and Stemmer, 1999), but with a faster rate (Patten et al., 1997; Stemmer, 1994).

The method involves the digestion with DNase I of a large Ab gene to create a pool of random DNA fragments. These fragments can then be reassembled into full-length genes by repeated cycles of annealing in the presence of DNA polymerase (Stemmer, 1994). The fragments prime each other based on homology, and recombination occurs when fragments from one copy of a gene prime another copy, causing a template switch. Because of the template switching events, a certain level of homology is required among the parental genes to be suitable for this method (Stemmer, 1994).

DNA shuffling offers several advantages over more traditional mutagenesis strategies. Compared to methods such as error-prone PCR and site-directed mutagenesis, DNA shuffling can be used with longer DNA sequences, and also allows for the obtention and selection of clones with mutations outside of the binding or active site of the protein, whereas site-directed mutagenesis is limited to a given region of the protein due to the limitation in library size that can be efficiently transformed to construct the mutant library (Stemmer, 1994).
DNA shuffling was first performed with 1 kb-long interleukin 1β genes. The genes were broken into 10- to 50-bp fragments and reassembled to their original size and function (Stemmer, 1994). Sequencing of clones following the shuffling process revealed that reassembly introduced point mutations at a rate of 0.7%, a rate similar to that of error-prone PCR. A similar method was used to shuffle β-lactamase genes, where three cycles of shuffling followed by selection yielded a mutant with 32,000-fold increase in enzymatic activity. Error-prone mutagenesis of the same genes resulted only in a 16-fold increase (Stemmer, 1994).

3.5.3.3 Staggered Extension Process (StEP)

DNA shuffling by staggered extension process was developed in 1998 by Zhao et al., (1998). StEP consists in the priming of template sequences followed by several cycles of denaturation and shortened annealing/polymerase-catalyzed extension. During each cycle, the DNA fragments can anneal to different templates based on sequence complementarity, and extend further to create recombinant genes. Because of the template switching, the growing polynucleotide chains can contain information from one or various parental clones (Zhao et al., 1998). The whole process can be performed in a single PCR reaction, and results in a pool of mutants, the majority of which are functional.

3.6 Expression and Purification

There is a growing demand for rAbs for treatment of human diseases, in vitro diagnostic tests, and affinity purification methods, placing pressure on current production capacity which is based largely on bacterial and tissue culture techniques. Alternative
systems, such as yeast (Horwitz et al., 1988), baculovirus (zu Putlitz et al., 1990) and plants (Hiatt and Ma, 1992), are very attractive, both for cost effectiveness and individual advantages such as scalability and safety for instance, that are absent in bacterial or mammalian systems. Several plant-produced Abs (plantibodies) are undergoing clinical trials and the first commercial approval could be only a few years away. The performance of the first generation of products has been very encouraging so far (Stoger et al., 2005). Ongoing studies are addressing further biochemical constraints, and aim to further improve yields, homogeneity and authenticity (e.g. its binding characteristics compared to parent Abs). There is no universal expression system that can guarantee high yields of recombinant product, as every Ab will pose unique challenges. The choice of an expression system will depend on many factors: the type of rAb being expressed, sequence of the individual rAb and the investigator’s preferences.

Given that the focus of this book is application of immunoassays within agricultural biotechnology, studies and experimental findings which describe mammalian expression and post-translational modification, etc. support rAbs as therapeutic treatments, will not be addressed.

3.6.1 Prokaryotic Systems

3.6.1.1 *E. coli* Cytoplasmic Expression

The expression of rAb fragments in the reducing environment of the cytoplasm of *E. coli* often leads to the formation of insoluble inclusion bodies, which contain unfolded protein (Verma et al., 1998). This necessitates the development of refolding protocols to recover functional Abs. In addition, accumulation of foreign protein in bacterial cells
may lead to their poor growth. Inducible promoters can, therefore, reduce the risk of cell

toxicity. *lac, trp, and their hybrid tac* promoters, which are regulated by the *lac* repressor
and induced by IPTG, are such examples. Another popular promoter is the λP<sub>L</sub> promoter

responsible for the transcription of the λ DNA molecule, which is regulated by a
temperature sensitive repressor. The T7 RNA promoter can also be used to obtain tightly
controlled, high level expression. Together with the T7 RNA polymerase (encoded by T7
*gene1*), it is one of the most powerful systems currently employed for recombinant
protein productions. The efficiency of T7 RNA polymerase in processing gene transcripts
is superior to that of native *E. coli* translation machinery which has made it a very
attractive for high level production of recombinant proteins (see review by Terpe, 2006).

Even under optimized expression conditions with a suitable promoter system,
cytoplasmic expression of functional rAbs and fusion proteins can be very challenging.
For example Peipp et al. (2004) reported that functional rAbs and fusion proteins was
only obtained in a minority of the cases for which it has been attempted.

3.6.1.2 *E. coli* Periplasmic Expression

Expression of Ab fragments into the periplasmic space of *E. coli* is the most
promising route to produce functional rAb fragments. This methodology was first
developed for Fv (Skerra and Plückthun, 1988) and Fab (Better et al., 1988; Plückthun
and Skerra, 1989) fragments. The periplasmic route is similar to protein synthesis
pathway in eukaryotes, in which the nascent proteins go through the endoplasmic
reticulum (ER) and into the Golgi apparatus. The transport of secretory proteins to the
periplasm of *E. coli* is comparable to that of ER and can lead to proper folding. Signal
sequences such as *pelB* (pectate lyase), derived from the *pelB* gene of *Erwinia carotovora* are added in frame with the genes encoding the H and L chains of Ab fragments, resulting in simultaneous secretion of both chains into the periplasmic space (Plückthun and Skerra, 1989).

The advantage of periplasmic secretion over cytoplasmic expression is that it leads to the production of an assembled, fully functional product without having to refold the protein *in vitro*. The oxidative environment of the periplasm allows for disulfide bond formation and the low protease environment permits stable folding of the recombinant proteins (Better et al., 1988). Both intra- and inter-chain protein folding and heterodimer associations occur in the periplasm, which is necessary for proper folding of a functional Ab (Plückthun and Skerra, 1989). The bacterial environment, however, is not efficient in producing full length Ab molecules.

### 3.6.2 Eukaryotic Systems

Production of Ab fragments in eukaryotes has also been widely studied, from unicellular yeast cultures (Freyre et al., 2000; Gach et al., 2007) to higher plants (Hiatt et al., 1989). In this section, different eukaryotic expression systems used for recombinant antibody production will be described and their individual pros and cons will be discussed.

#### 3.6.2.1 Yeast Expression

The main advantages of yeast over bacterial systems are related to the fact that yeast is both a microorganism and a eukaryote. Frenken et al. produced both scFv (1998) and V_{H}H (2000) in *E. coli* and *Saccharomyces cerevisiae* and found that V_{H}H production
levels in the latter organism was up to 100 mg·L$^{-1}$, i.e., 1000-fold higher than $E. \text{coli}$. Furthermore, $E. \text{coli}$-produced $V_{\text{H}}$H exhibited less specific activity (higher fraction of incorrectly folded soluble species present in the periplasmic preparation) than yeast. In another study (Huang and Shusta, 2006), two scFvs were expressed at 0.25 mg·L$^{-1}$ in $E. \text{coli}$, while yields in $Pichia \text{pastoris}$ were between 60 - 250 mg·L$^{-1}$.

Yeast provides folding pathways for heterologous proteins and, when yeast signal sequences are used, correctly folded proteins can be secreted into culture medium. Moreover, yeasts rapidly grow on simple growth media, and, therefore, represent an attractive option for industrial-scale production of rAbs. Proteins which may accumulate as insoluble inclusion bodies in $E. \text{coli}$ are often soluble when expressed in yeast (Freyre et al., 2000). In addition, the degradation of heterologous proteins, often a problem in $E. \text{coli}$, is usually reduced in yeast. Another unique feature of yeast is that it has the option of stable transformation without the use of antibiotic resistance, like ampicillin or kanamycin resistance commonly used with $E. \text{coli}$. $E. \text{coli}$ uses episomal vectors which propagate extra-chromosomally, whereas in yeast, the vector carrying the gene of interests is integrated into the host genome by homologous recombination (Joosten et al., 2003).

Secretion of functional Fab and whole immunoglobulin (IgG) Abs in yeast was first demonstrated by Horwitz et al. (1988) in $S. \text{cerevisiae}$, although expression levels were rather low (approx. 200 ng Fab·mL$^{-1}$). Many efforts have been devoted to improving the expression level of Ab in yeast cells. Yeast secretion yields have been reported to be as high as 1.2 g·L$^{-1}$ for scFv fragment in fed batch fermentation cultures (Freyre et al., 2000). Elements that control the expression level of foreign protein in yeast have been
well studied. The yeast invertase signal sequence, the PGK promoter and the polyadenylation signal were used in the study of Fab and IgG expression (Horwitz et al., 1988). The proteins were correctly folded and whole Ab and Fab were purified from the culture supernatants. The antibodies behaved indistinguishably from their lymphoid cell-derived counterparts in both direct and competition binding assay. The chimeric whole Ab from yeast exhibited the same ADCC activity as the chimeric Ab from Sp2/0 cells. Codon optimization and lower repetitive AT and GC content also improve the expression levels of some rAbs by 5- to 10-fold in standard shake flask cultures (Sinclair and Choy, 2002; Woo et al., 2002), but this is not the case for all rAbs (Ouchkourov et al., 2002).

Non-conventional species of yeasts and filamentous fungi have been tested for expression of Abs and rAb fragments. Abdel-Salam et al. (2001) reported the expression of Fab in *Hansenula polymorpha*. Nevertheless, Ab monomers (kappa and gamma chains) did not assemble into a heterodimer and were poorly secreted. *Yarrowia lipolytica* and *Kluyveromyces lactis* have also been reported for scFv production (reviewed by Joosten et al., 2003). Filamentous fungus *Trichoderma reesei* was also reported to have secreted 1 mg·mL\(^{-1}\) of Fab into the growth medium under shake-flask conditions and the yield could be increased to 150 mg·L\(^{-1}\) in bioreactor cultures by fusing the heavy Fv chain to *T. reesei* cellubiohydrolase I (CBHI) enzyme (Nyyssönen et al., 1993; Nyyssönen and Keränen, 1995).

A strong preference has been given to *P. pastoris* for its aerobic growth; a key physiological trait that greatly facilitates culturing at high cell densities compared with the fermentative baker’s yeast *S. cerevisiae* (Cregg et al., 1993). An scFv-green
fluorescent protein was expressed by methylotrophic yeast *P. pastoris* under the methanol-inducible alcohol oxidase 1 (AOX1) promoter and secreted into the growth medium. The soluble fusion protein was properly folded without additional renaturation or solubilization (Petrausch et al., 2007). Rahbarizadeh et al. (2006) found that high inoculum densities limit growth potential but gave rise to a higher level of V_{H}H production in *P. pastoris*. Medium composition and pre-induction osmotic stress were found to have the greatest influence on yield, but can be improved when casamino acid or EDTA are included in growth medium. However, glycerol supplementation during induction resulted in increased growth rates and biomass accumulation, but expression of scFv was repressed (Hellwig et al., 2001). Gasser et al. (2006) first engineered the protein disulfide isomerase (PDI) and the unfolded protein response (UPR) transcription factor HAC1 to be constitutively over-expressed in *P. pastoris* (Mattanovich et al., 2004). While the over-expression of HAC1 led to a moderate increase of Fab secretion, i.e., 1.3-fold, PDI enabled an increase of the Fab secretion level by 1.9 fold. Hence, the formation of interchain disulfide bonds can be seen as a major rate limiting factor to Fab assembly and subsequent secretion.

### 3.6.2.2 Insect Cell Expression

Insect cell expression systems have been shown to be a viable alternative to standard microbial and mammalian systems designed to express rAb fragments and full-size mAbs, particularly for Abs designed for use as therapeutic proteins (Demangel et al., 2005; Guttieri et al., 2000; Verma et al., 1998). The baculovirus-mediated gene expression in insect cells not only produces large amounts of the foreign protein while
allowing it to retain its functional activity, it has a highly restricted host range and is less likely to have contamination during the downstream process and cause harmful effects in end users. Between 1 to 500 mg of recombinant protein per liter of infected cells have been reported (Filpula, 2007; Guttieri et al., 2000). However, the success of a foreign gene expression in insect cells depends on a number of factors. High quality growth media and careful culturing is required and, for optimal results, the insect cells should be highly viable and in the log phase of growth. Unfortunately, expression of the foreign protein is controlled by a very late viral promoter and peaks while the infection culminates in death of the host cell, thereby allowing for only transient expression of the rAb. Previous studies also suggested that, during the late phase of baculovirus infection, the host’s secretory pathway can become impaired (Reavy et al., 2000).

An alternative to baculovirus-mediated expression is based on stable transformation of the gene, under the control of an appropriate promoter, into insect cells. For example, the Drosophila metallothionein promoter has been used to control expression and found to be tightly regulated, directing high levels of transcription when induced by heavy metals, such as cadmium or copper (Guttieri et al., 2000). Moreover, the polyhedrin promoter is considerably stronger than most other eukaryotic promoters, and therefore enables the gene to be transcribed at a high level, causing recombinant proteins to be secreted in the insect cell culture in large amounts (Tan and Lam, 1999). To allow for the continuous expression of Ab genes, Ab DNA can be inserted into insect cell chromosomes under the control of the baculovirus immediate to early gene promoter (IEI), which is recognized by the insect cell RNA polymerase (Guttieri et al., 2000).
concentration of IgG recovered from the transformed insect cell culture medium was considerably lower (approx. 0.06 µg of IgG·mL⁻¹) than the level generated by infection with the baculovirus recombinant (approx. 9 µg of IgG·mL⁻¹) and with the predicted yield from hybridoma cells (1 – 10 µg of IgG·mL⁻¹). Nevertheless, the baculovirus system is still considered a viable alternative to microbial expression systems for whole Ab molecule expression and when post-translational modification, e.g. glycosylation, is required.

3.6.2.3 In planta Expression

“Plantibodies”, a contemporary term to describe full size Ab or rAb fragments expressed in plant tissues, was first reported almost twenty years ago by Hiatt et al., (1989). Since then many researchers have been trying to express and scale up the production of Ab or fragments in plants (reviewed within Fischer et al., 1999; Peeters et al., 2001; Stoger et al., 2002). Using plants as bioreactors presents many advantages related with manufacturing facilities, production costs and biosafety, among others. However, despite the production costs reduction and the biocomparability of plantibodies with their conventional Ab counterparts, contamination risk exists with mycotoxins, alkaloids, allergenic and immunogenic proteins, especially if the expression host is a tobacco plant. However, the favorable properties of plants are likely to make them a useful alternative for small, medium and large scale production throughout the development of new Ab-based pharmaceuticals or diagnostic reagents. For example, Khoudi et al. (1999) reported the production of a functional, purified anti-human IgG mAb through expression of its encoding genes in perennial transgenic alfalfa.
The deposition and storage of a scFv rAb fragment in pea seeds was reported using a seed specific USP promoter (Saalbach et al., 2001), in cereals (Stoger et al., 2000), tobacco plants (Ko and Koprowski, 2005) or suspension cells (Yano et al., 2004). In particular, seeds offer special advantages, such as ease of handling and long-term storage stability. Nevertheless, most of the plantibody studies have focused on expression in leaves. Arguably, there may not be a high enough demand for diagnostic Ab that requires the large-scale Ab production possible within plant expression, nonetheless, the idea of expressing functional rAb molecules in plant has already provided a new way of understanding plant/virus interaction (Sudarshana et al., 2007). The studying and/or altering the function of an antigen in vivo, also termed immuno-modulation, has also gained new insights into plant physiology (Artsenko et al., 1995; 1998; Conrad and Fiedler, 1998; Tavlodoraki et al., 1993), plant pathogen resistance (Peschen et al, 2004) or potentially confers herbicide resistance to plants (Almquist et al., 2004; Weiss et al., 2006).

Currently, Agrobacterium and particle bombardment are the most commonly used technologies for plant transformation. A single plant binary vector carrying genes encoding heavy and light chains under two different promoters has been used to express both genes and to assemble functional full-size Ab (Düring et al., 1990). The major drawback of the binary vector is low transformation efficiency due to the large size of the Ab DNA, in addition to the enormous binary vector required (Komori et al., 2007). Instead of molecularly stacking the heavy and light Ab genes in the same vector, crossing two transgenic lines separately expressing the heavy and light chain provides an
alternative that is less demanding in terms of molecular biology (Schillberg et al., 2003). The particle bombardment approach can insert genes into the genome of the plant nucleus or the plastid genome, depending on the vector construct. Chloroplasts have been used for stable expression of mAb in the chloroplast genome. Advantages of targeting the chloroplast genome include no position effect, no gene silencing, high expression/accumulation, and minimized environmental concerns (Daniell et al., 2004). Chloroplasts can process proteins with disulfide bridges, which is required for proper folding of proteins. Although stably transformed (transgenic) plants are able to express correctly folded and functional Ab of both the IgG and IgA classes (Ma et al., 1995) or its fragments (Owen et al., 1992), yields are generally very low (usually in the range of 1-40 µg·g⁻¹ of fresh biomass). In addition, the time necessary to generate the first grams of research Ab material is very long, requiring > 2 years (Giritch et al., 2006).

To shorten the time required to obtain the functional antibodies from plants, transient expression using agro-infiltration or plant viral vector system have been used (Giritch et al., 2006; Ko and Koprowski, 2005). Using the subgenomic promoter of TMV coat protein and tobacco mild green mosaic virus variant U5 coat protein, two separate virus vectors were used to co-infect Nicotiana benthamiana leaf cells. Full length heavy chain and light chain proteins were produced and assembled into glycosylated functional full size Ab (Verch et al., 1998). Regeneration time is much shorter than that for stable transformation, and different host plants can be infected by the same viral vector, allowing time-efficient screening for recombinant gene expression. More importantly, it
saves the labor-intensive step of crossing different transgenic plants producing different Ab subunits.

Many different Ab formats have been expressed successfully in plants. These include full-size antibodies (Giritch et al., 2006; Ma et al., 1995), camelid heavy-chain antibodies (Ismaili et al., 2007), Fab fragments (Weiss et al., 2006; Yano et al., 2004), and scFvs (Owen et al., 1992; Saalbach et al., 2001; Stoger et al., 2000). Regardless of which form of Ab fragments are being produced by the plant systems, ER is an important site of the major bio-functions of synthesis, assembly, and glycosylation of these protein molecules. Antibodies are often targeted to subcellular compartments or the apoplastic space since most proteins are more stable in the subcellular compartment than in the cytosol. The greatest accumulation of full size antibodies has been obtained by targeting to the apoplastic space and the greatest accumulation of scFv antibodies was obtained when the antibodies were retained in the ER (Daniell et al., 2004).

The average yield of the recombinant protein using plant secretion sequence (SS) is usually 0.1 - 2% of total soluble protein (Yano et al., 2004). So far, three different leader sequences have been tested: human derived leader sequence (LS), dicotyledonous calreticulum derived SS, and monocotyledonous hordothionin derived SS. The latter did not consistently result in mAb expression, while plants transformed with the dicot SS construct grew more vigorously and expressed the antibodies more consistently than transgenic cells made with human LS. The promoter is another critical element determining the expression level, for examples, Cauliflower Mosaic virus 35S (CaMV35S) promoter for dicotyledonous plants (McLean et al., 2007; Olea-Popelka et
Petruccelli et al. (2006) successfully produced a full length anti-rabies mAb molecule in transgenic tobacco using CaMV35S to drive the heavy chain, while the potato proteinase inhibitor II (pin2) promoter controlling light chain gene produced functional mAb. An effective plant production system for recombinant Ab fragments also requires the appropriate control of post-translational processing of recombinant products. The ER retention signal sequence (KDEL) has little effect on the accumulation of Ab in the transgenic leaves, but leads to higher Ab yields in seeds. The proteins purified from leaves contain complex N-glycans, including Lewis a epitopes, as typically found in extracellular glycoproteins and consistent with an efficient ER retention and the cis-Golgi retrieval of the Ab. The glycosylated proteins purified from the seed were partially secreted and sorted to protein storage vacuoles (PSVs) in seeds and not found in the ER (Ramirez et al., 2003). More importantly, full size antibody (McLean et al., 2007) or scFv fragments (Almquist et al., 2004, 2006; Makvandi-Nejad et al., 2005) produced in Tobacco have been proved to be functional. This further supports the fact that “plantibodies” could be an alternate source of diagnostic as well as therapeutic antibodies.

3.7 Application to Immunoassays and Agricultural Biotechnology

3.7.1 Immunoassays

Given the widespread adoption of genetically modified (GM) crops within global agriculture and strict regulations governing their co-existence with non-GM crops (Demont and Devos, 2008), mandatory product labeling (e.g., CODEX alimentarius, EU, 2003; etc.) and international trade, there is an increased need to standardize and
continuously upgrade the analytical methods used to monitor and verify the presence of GM traits within food and feed (Dong et al., 2008).

Immunoassays for the detection of protein expression within GM crops are currently limited to enzyme linked immunosorbent assays (ELISA) based on either trait-specific polyclonal serum or mAbs, as well as rapid lateral-flow / immuno-chromatographic diagnostic strip kits (typically based on polyclonal serum) (e.g., Lin et al., 2001; Ma et al., 2005; Tripathi, 2005; Van den Bulcke et al., 2007). Both immunoassay formats are widely used in the US, Canada, and other countries which produce and export GM crops as a primary method for determining minimum thresholds of GM products within commercial food and feed shipments (Stave, 2002).

A wide array of immunoassay products are currently available to assess GM expression within agricultural crops. These testing formats are developed within private, or in-house programs or are available as commercial products from companies such as: Agdia Inc. (Elkart, IN, USA), EnviroLogix Inc. (Portland, ME, USA), and Strategic Diagnostics Inc. (Newark, DE, USA). Although these platforms are very useful qualitative tools to detect the presence of GM traits within commercial crops, the results generated by current immunoassay products are often limited by detection limit and overall precision, or consistency, of results. For example, Ma et al. (2005) reported that both ELISA and DNA-based (PCR) tests were capable of distinguishing samples with GM concentrations between 0.1 to 0.5%, but the precision at this range was very low as results were highly inconsistent. Another consideration is the disparity between the time, costs, and resources required to develop GM-specific immunoassay reagents based on
traditional mAb and polyclonal platforms relative to the rapid progression of GM crops
toward newer events based upon multiple / stacked traits.

Antibody engineering has evolved rapidly over the past 20 years to the point where
it is possible to isolate and optimize high-affinity and Ag-specific rAb fragments as a
cost-effective alternative to established mAb technologies (biomedical applications
reviewed in Holliger and Husdon, 2005). In this regard, rAb fragments (i.e., Fab, scFv,
V_{H}H) could represent a means to complement, and potentially displace, polyclonal serum
and mAbs used within immunoassay formats. In fact, one could argue that the
application of rAb technology to detect GM proteins within plant matrices would be a
natural extension of published research which demonstrated effective use of rAb
technology within immunoassay formats to detect ligands ranging from: mycotoxins
(Wang et al., 2007; 2008) to caffeine (Ladenson, 2006) to large complex protein toxins
such as cholera toxin, ricin and staphylococcal enterotoxin B (Goldman et al., 2006). The
application of rAb technology within current ELISA and lateral flow stick GM
immunoassay formats would obviously depend on any benefits, or advantages, imparted
by this new technology. The following is an initial (and by no means exhaustive) list of
“key traits” that rAb fragments could exhibit relative to current mAb and polyclonal
technology: enhanced GM trait specificity based on recognition of a single target protein;
improved sensitivity to target protein; decreased development costs and timelines; ability
to combine rAb reagents to enable simultaneous and customized detection of stacked GM
traits; improved durability and speed of deployment impart convenient and real-time
immunoassay results.
Apart from the advantages associated with improved affinity, cost-effective expression and Ag-specificity (as discussed within this chapter), rAb technology also imparts unique opportunities to optimize the physical characteristics of rAb fragments to suit the intended use pattern. For example, it is possible to express single-domain V_{H} fragments in a multimeric pentamer format as a means of increasing antibody avidity (Zhang et al., 2004) with potential use in lateral flow devices and ELISA kits. Depending on the intended application and immunoassay design, it is also possible to couple rAb fragments to larger indicator molecules (e.g., fluorophores, etc.) to impart visual GM detection in real time. Finally, given that V_{H} fragments have been shown to have high affinity for large, complex enzymes (e.g., Desmyter et al., 1996; 2002), such fragments may be particularly suited for detection of GM traits which express enzymes which confer tolerance to the herbicide glyphosate (e.g., GAT, EPSPS, etc.).

3.7.2 Research Applications to Agricultural Biotechnology

Apart from standard immunoassay detection procedures, it is also possible to apply rAb technology to study structure-function relationships between GM traits and the target organism. For example, Fernández et al. (2008) reported the use of scFv phage-display libraries and various bio-panning techniques to characterize epitopes that mediate binding of Cry1Ab and Cry11Aa toxins with target protein receptors within Manduca sexta and Aedes aegypti, respectively. Such results could provide insights into the mechanism of insect specificity and mode of action of Cry toxins to enable strategies designed to improve target insect toxicity and specificity. Ongoing research in this area could be useful to study, and potentially improve upon, the precise mechanism and toxicity by
which target transgenic events work within the host crop and efficacy versus the target organism. Finally, rAbs are also well suited to study and modify metabolic pathways within plants as a means of developing new transgenic traits and GM products (reviewed in Nölke et al., 2006).
RESEARCH CHAPTER 1: CLONING, EXPRESSION, AND CHARACTERIZATION OF A SINGLE DOMAIN ANTIBODY FRAGMENT WITH AFFINITY FOR 15-ACETYL-DEOXYNIVALENOL.

4.1 Abstract

A single-domain variable heavy chain (V_H) antibody fragment specific to the mycotoxin 15-acetyldeoxynivalenol (15-AcDON) was obtained after immunization of a llama (Llama glama) with the protein conjugate 15-DON-BSA plus TiterMax™ Classic adjuvant. After confirmation of a polyclonal response to DON toxin in both conventional (cIgG) and heavy chain antibody (HCAb) fractions, a V_H library was constructed from amplified cDNA by nested PCR. V_H fragments with binding affinity for the mycotoxin were selected by panning of the phagemid library against microtiter plates coated with 15-DON-OVA. The dominant clone (NAT-267) was expressed in E. coli and was purified as a V_H monomer (mNAT-267) at a final concentration of 1.3 mg·mL⁻¹. Isolated NAT-267 V_H DNA was fused to the homopentamerization domain of the B subunit of verotoxin to generate the pentabody format of single-domain antibody (sAb). The V_H pentamer (pNAT-267) was expressed in E. coli and was purified at a final concentration of 1.0 mg·mL⁻¹. Surface Plasmon Resonance (SPR) analysis of soluble mNAT-267 binding kinetics to immobilized 15-DON-HRP (Horse Radish Peroxidase) indicated a dissociation constant (K_D) of 5 μM. Competitive Direct Enzyme-Linked Immunosorbent Assay (CD-ELISA) and Fluorescence Polarization Assay (FPA) inhibition experiments with monomer and pentamer confirmed binding to 15-AcDON. Competitive inhibition FPA tests with mNAT-267 and pNAT-267 IC⁵₀ values for 15-AcDON hapten were 1.24 μM and 0.50 μM, respectively. These values were similar to the IC⁵₀ value of 1.42 μM for 15-AcDON given by polyclonal llama serum sampled 56 days after immunization.
Competition formats for structurally related trichothecenes resulted in no cross reactivity to: DON; 3-acetyldeoxynivalenol (3-ADON); neosolaniol (NEO); diacetoxyescirpenol (DAS); and T-2 toxin. Our study confirmed that recombinant V_{H}H fragments capable of binding low molecular weight haptens can be produced through the creation and panning of hyper-immunized single domain (sdAb) libraries.

Key Words

V_{H}H, Hapten, Mycotoxin, 15-acetyl-deoxynivalenol, Phagemid Library, Surface Plasmon Resonance, CD-ELISA, Fluorescence Polarization Assay.

4.2 Introduction

Deoxynivalenol (3,7,15-trihydroxy, 12,13-epoxy-trichothece-9-en-8-one), or DON, is one of many members of the trichothecene group of mycotoxins produced by Fusarium species. DON is a low molecular weight hapten (M.W. 296 Da) containing one primary and two secondary hydroxyl groups conferring limited solubility in water and high solubility in various polar solvents such as methanol and acetonitrile (Desjardins, 2006). Of the trichothecene mycotoxins that accumulate within cereal grain, DON toxin and its acetylated metabolites (i.e., 15-acetyl-deoxynivalenol (15-AcDON) and 3-acetyl-deoxynivalenol (3-AcDON)) are considered among the most commonly and widely distributed food and feed contaminants (Abramson et al., 2001; Greenhalgh et al., 1986).

Immunoassay techniques using polyclonal and monoclonal antibodies (mAb) have been developed to detect DON and other mycotoxins within grain, food, feed, and animal serum (e.g., Maragos and McCormick, 2000; Sinha et al., 1995). These systems typically use anti-DON mAbs from hybridoma cell lines producing murine immunoglobulins.
Although these tests provide robust and precise results, mAb development requires highly specialized equipment and labor-intensive procedures to select, culture, and maintain optimal hybridoma cell lines (Sinha et al., 1995).

Heavy chain antibodies found within sera of the camelidae (i.e., camels, dromedaries and llamas) (Hamers-Casterman et al., 1993) are unique in that they are devoid of the variable light chain (V\textsubscript{L}) and first constant region (C\textsubscript{H1}) which are common to larger conventional immunoglobulin (cIgG) structures (Nguyen et al., 1999). The paratope of HCAbs is comprised of a single heavy chain variable domain (often called ‘V\textsubscript{H}H’) which is directly linked to the second constant domain (C\textsubscript{H2}) by a unique hinge region (Hamers-Casterman et al., 1993).

Extended hinge regions coupled with deletion of the C\textsubscript{H1} domain, and amino acid substitutions within the variable domain are believed to impart increased hydrophilicity and solubility of HCAbs within serum (Desmyter et al., 2001; Nguyen et al., 2000; Vu et al., 1997). These properties coupled with expanded complementarity determining region (CDR) loops within the V\textsubscript{H}H paratope may expand the antigen (Ag) - binding repertoire within a cameld immune system by enabling HCAbs to bind otherwise inaccessible epitopes (Muylldermans, 2001; Nguyen et al., 2000).

Despite their lack of variable light and heavy chain pairing, single domain antibodies (sdAb) recognize and bind a wide variety of epitopes and Ag types (Nguyen et al., 2000; Sheedy et al., 2006; van der Linden et al., 2000). Recombinant sdAb fragments derived from phage libraries are regarded as among the smallest intact Ag-binding fragments currently known (Muylldermans, 2001) and demonstrate robust and stable
expression in bacteria (e.g., Desmyter et al., 2001; Ghahroudi et al., 1997; Sheedy et al., 2006; etc.) and yeast (e.g., Dolk et al., 2005; Frenken et al., 2000; etc.) relative to other rAb formats (e.g., Fab, Fv, scFv, mAb, etc.).

The advantages associated with ease of expression, stability, and solubility of V_{H}H fragments can be attributed to key differences relative to standard rAb fragments (e.g., scFv, Fv, Fab, etc.). V_{H}H fragments are small (≈ 14 – 15 kDa), typically have hydrophilic amino acid substitutions within the second framework region (FR-2) and by their single-domain nature, do not require the linker associated with the standard scFv format which can lead to problems associated with aggregation, proteolytic degradation and variable expression within recombinant hosts (Arbabi-Ghahroudi et al., 2005). Improved functionality over various temperature and solvent regimes (Goldman et al., 2006; van der Linden et al., 1999) coupled with high levels of recombinant expression combine to make V_{H}H fragments useful reagents within next-generation immunoassays (Goldman et al., 2006, Ladenson et al., 2006) and for expression within recombinant hosts to evaluate potential in vivo mechanisms of immunomodulation (Muylldermans, 2001).

As with other rAb fragments, V_{H}H fragments isolated from hyper-immunized or naïve libraries are highly specific based on the recognition of unique epitopes on target Ags (Ghahroudi et al., 1997; Goldman et al., 2006; Ladenson et al., 2006; Sheedy et al., 2006; van der Linden et al., 2000). A wide-range of affinities has been published for Ags ranging from large immunogenic proteins to enzymes to low-molecular weight haptens (reviewed in Muylldermans, 2001). A growing number of V_{H}H fragments have been isolated which demonstrate a range of binding affinities against an array of different
hapten types (Alvarez-Rueda et al., 2007; Goldman et al., 2006; Ladenson et al., 2006; Sheedy et al., 2006; Spinelli et al., 2000; 2001; Yau et al., 2003b). These results serve to validate potential uses of this new technology in terms of small molecule binding.

The objective of this research was to isolate and characterize mycotoxin-specific recombinant V\textsubscript{H}H fragments from a hyper-immunized phage-display library. The identification of mycotoxin-specific fragments will provide additional information to further characterize the nature and binding kinetics of V\textsubscript{H}H antibody fragments to haptens. Such fragments will also aid in the development of low cost, durable reagents required for effective and robust toxin detection systems (Goldman et al., 2006; Ladenson et al., 2006). Finally, with favorable binding affinity and expression, mycotoxin-specific V\textsubscript{H}H fragments can also be used in eukaryotic systems in vivo (e.g., yeast and plant cells) to evaluate their potential for immunomodulation of the cytotoxic effects of DON and related trichothecene mycotoxins.

4.3 Materials and Methods

4.3.1 Reagents and Solutions

Restriction enzymes were purchased from New England Biolabs (Mississauga, ON). Ampicillin, kanamycin, bacto-tryptone, bacto-yeast extract, isopropyl β-D-1-thiogalactopyranoside (IPTG), phenylmethylsulphonyl fluoride (PMSF), isopropanol, 6-aminofluorescein (fluoresceinamine isomer II), bovine serum albumin (BSA), ovalbumin (OVA), TiterMax\textsuperscript{TM} Classic, N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid (HEPES), Tris HCl, sodium dodecyl sulfate (SDS), triethylamine, polyethylene glycol (PEG), and polyoxyethylene sorbitan mono-laurate (Tween 20) were purchased from
Sigma Chemical Co. (St. Louis, MO). Skim milk was purchased from Difco (Oakville, ON). PCR primers were synthesized by Gibco BRL (Burlington, ON). Mouse anti-6-His monoclonal IgG and goat anti-mouse alkaline phosphatase conjugate were purchased from Qiagen (Mississauga, ON). PVDF membrane and NBT (nitro blue tetrazolium chloride) / BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) were purchased from Roche Diagnostics (Laval, PQ). 96 well Maxisorp™ microtiter-plates were purchased from Nalge Nunc Inc. (Naperville, IL). Rabbit anti-6-HIS-HRP conjugate was purchased from QED Bioscience Inc (San Diego, CA). AP and HRP development substrates were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

4.3.2 Immunogen and Llama Immunization

All DON-based reagents were obtained from the Eastern Oilseed and Cereal Research Center (ECORC) of Agriculture and AgriFood Canada (AAFC) Ottawa, Ontario, Canada. DON was isolated from large-scale fermentations of Fusarium graminearum liquid culture followed by purification as described by (Greenhalgh et al., 1986). 15-AcDON was prepared by selective hydrolysis of 3,15-diacetyl-DON, which was obtained by acetylation of DON or 3-AcDON (Savard, 1991). Neosolaniol and T-2 Toxin were isolated according to standard methods (Greenhalgh et al., 1986). Diacetoxyscirpenol (DAS) toxin was purchased from Sigma-Aldrich (Oakville, Ontario). Synthesis of DON-protein conjugates 15-DON-BSA, 15-DON-OVA, 3-DON-HRP and 15-DON-HRP was based on standard methods (Bauminger and Wilcheck, 1980) with modifications as previously described (Sinha et al., 1995). DON-protein conjugates were
purified by size exclusion chromatography on Sephadex G-15 (2.5 x 21-cm column) with distilled deionized water at a flow rate of 4 mL·min⁻¹.

A two-year-old male llama was immunized by sub-cutaneous (Sub-Q) lower-back injection of 15-DON-BSA (250 μg·mL⁻¹) conjugate in 1.0 mL PBS + 1.0 mL of TiterMax Classic adjuvant. The llama was boosted with a second injection three weeks after the first immunization followed by three subsequent injections every two weeks for a total of five immunizations over a nine-week period.

Pre-immune serum was collected as a negative control, and 15.0 mL of blood was collected one week after each immunization to measure titer at 7, 28, 42, 56 and 70 days after the first immunization. Serum was prepared by storing blood samples overnight at 4°C followed by centrifugation at 2,700 x g for 10 minutes at 4°C. Samples of fresh blood (20 mL) were collected one week after the third and fifth immunizations from which leukocytes were isolated, i.e., 42 and 70 days after immunization, respectively. The isolation of RNA from peripheral leukocytes was performed using a QIAamp RNA Blood Mini Kit (Cat.No. 52304 – Qiagen Inc., Mississauga, Canada).

4.3.3 Fractionation of Polyclonal Serum

Llama serum from a pre-immune sample and from samples taken one week after the third and fifth immunizations (42 and 70 day samples, respectively) were fractionated by protein G and protein A chromatography (Hi Trap, Pharmacia, Upsala, Sweden) and separated by acidic elution as previously described (Hamers-Casterman et al., 1993). Serum fractions A1 (HCAb), A2 (HCAb), G1 (HCAb), and G2 (cIgG) from each collection date were dialyzed against PBS and subsequently stored at 4°C with 0.01%
NaN\textsubscript{3} as a preservative. The IgG content of the isolated serum fractions was measured using the BCA-protein assay (Pierce, Rockford, IL, USA).

**4.3.4 Confirmation of Titer**

Polyclonal serum was screened against 15-DON-OVA or OVA (control) by indirect ELISA as based on a modified standard protocol (Sinha et al., 1995). Briefly, a standard 96-well microtiter plate was coated with 100 μL 15-DON-OVA (5 μg∙mL\textsuperscript{-1} in 0.15 M carbonate-bicarbonate buffer, pH 9.6). Blank wells were coated with 100 μL OVA (20 μg∙mL\textsuperscript{-1} in PBS). After incubation overnight, plates were washed with PBST, and blocked with 1.0% OVA (2 hours, 37°C) followed by washing with PBST. Non-immunized (control) wells for 15-DON-OVA and OVA (blank) were coated with pre-immune polyclonal serum. Treatment wells were coated with 42- and 70-day serum samples. Immune and non-immune polyclonal sera were diluted serially from 1:3 to 1:59,049 in PBS for both 15-DON-OVA and OVA (blank) coated wells. After incubation at room temperature (1.5 hours) and washing with PBST, 100 μL of goat anti-llama IgG (1:1,000 dilution PBS) was added. Plates were incubated at 37°C for 1 hr and washed five times with PBST. Swine anti-goat HRP conjugate (100 μL; 1:3,000 dilution in PBS) was added followed by incubation and washing as described. Titer was determined with standard colourimetric buffer followed by 1 M H\textsubscript{3}PO\textsubscript{4} and measurement of optical density at 450 nm on a standard ELISA plate reader (Dynatech MR5000). Best titers were defined as the greatest sample dilution giving twice the background absorbance of OVA control.
Titer of cIgG and HCAb fractions were tested against 15-DON-OVA or OVA (control) by indirect ELISA as described above with one modification. As an additional blocking step, equal volumes and concentrations of processed cIgG and HCAb fractions were ‘pre-incubated’ with 2% OVA in PBS. Antibody fractions were added to 15-DON-OVA and control wells and diluted in PBS to establish a dilution series from 1:2 to 1:1,024 and processed according to the previously described method (Sinha et al., 1995).

4.3.5 Library Construction

Total RNA was isolated from the leukocyte fraction of Peripheral Blood Lymphocytes (PBL) of whole blood samples collected one week after the third and fifth immunizations (42 and 70 days, resp.) using a QIAamp RNA Blood Mini Kit (Cat. No. 52304 – QIAGEN Inc., Mississauga, Canada). EDTA (dipotassium salt) at 1-2 mg ml⁻¹ blood was used as an anticoagulant. The V₃H library was constructed according to Ghahroudi et al., (1997) with minor modifications. Briefly, first strand complementary DNA (cDNA) was derived from 33 µL of total RNA (approx. 3 µg·20 µL⁻¹) isolated from 42 and 70 day PBLs using oligo dT primer and a ‘First-Strand cDNA Synthesis Kit’ (Amersham Biosciences, Buckinghamshire, UK). Resultant synthesis of V₃H and V₃H dsDNA occurred through nested PCR using proofreading Taq polymerase with sense and anti-sense hinge-specific primer mixtures “MJ1.2.3 Back”, and “CH₂ + CH₂b₃”, respectively (Table 4.1). After amplification, doublet PCR bands between 620 – 670 bp corresponding to V₃H – C₃H2 hinge HCAb dsDNA were agarose gel-purified. Gene-specific primers: MJ7 (Sense) and MJ8 (Antisense) (Table 4.1) were used to amplify the
V_H region of HCAb and add two SfiI restriction sites flanking the 5’ and 3’ termini of V_H coding sequences. PCR products were then pooled and agarose gel-purified.

Table 4.1 Nucleotide sequences of primers for PCR amplification of HCAb V_H genes.

<table>
<thead>
<tr>
<th>Type</th>
<th>Name</th>
<th>Nucleotide Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Isolation and Amplification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward MJ1.2.3</td>
<td>MJ1 Back</td>
<td>5’-GCCCGGCGCGAGCGCGCGCCATGGC-3’</td>
</tr>
<tr>
<td></td>
<td>MJ2 Back</td>
<td>5’-GCCCGGCGCGAGCGCGCGCCATGGC-3’</td>
</tr>
<tr>
<td></td>
<td>MJ3 Back</td>
<td>5’-GCCCGGCGCGAGCGCGCGCCATGGC-3’</td>
</tr>
<tr>
<td>Reverse CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>MJ13RP</td>
<td>5’-GCCCGGCGCGAGCGCGCGCCATGGC-3’</td>
</tr>
<tr>
<td></td>
<td>CH&lt;sub&gt;2b3&lt;/sub&gt;</td>
<td>5’-GCCCGGCGCGAGCGCGCGCCATGGC-3’</td>
</tr>
<tr>
<td>Addition of SfiI</td>
<td>Forward MJ7</td>
<td>5’-CATCGATATGACCGGCGCGGCGGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse MJ8</td>
<td>5’-CATCGATATGACCGGCGCGGCGGC-3’</td>
</tr>
<tr>
<td>Colony PCR</td>
<td>Forward PN2</td>
<td>5’-CCCTCATAGTAAACGATCT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse MJ13RP</td>
<td>5’-CCCTCATAGTAAACGATCT-3’</td>
</tr>
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<sup>a</sup> Key to symbols: M=A+C, K=G+T, S=G+C.

V_H dsDNA was ligated into pMED1 phagemid vector (Ghahroudi et al., 1997) using the LigaFast Rapid DNA ligation system (Promega) according to standard methods. Briefly, amplified V_H fragments were digested with SfiI restriction enzyme along with pMED1 phagemid DNA overnight at 50°C. To reduce the probability of self-ligation of the phagemid vector and to improve the functional size of the V_H library, pMED1 DNA was further digested with PstI and XhoI restriction enzymes (2 hours, 37°C). Once the ratio of insert to vector was optimized, V_H leukocyte DNA was ligated into the pMED1 phagemid vector at the SfiI restriction site prior to ligation to create the library.
After transformation into TG1 *E. coli* (Stratagene), *V*\(_{\text{H}}\)*H* library size and diversity were estimated by plating on LB + ampicillin (100 mg·mL\(^{-1}\)) agar plates followed by colony PCR using universal reverse primer “MJ13RP” and forward primer “PN2” (Table 4.1). 30 positive clones which produced a PCR product of approximately 600 bp (400 bp *V*\(_{\text{H}}\) plus 200 bp oligo DNA) were nucleotide sequenced. Positive clones were also digested with the random 4-mer restriction endonuclease enzyme *Bst*N1 followed by agarose gel electrophoresis. Clones were regarded as unique if the pattern of digestion produced a DNA ‘fingerprint’ that was different from other randomly selected clones.

4.3.6 *V*\(_{\text{H}}\)*H* Library Panning

The *V*\(_{\text{H}}\)*H* library was subjected to four rounds of panning on 96-well Maxisorp plates. Target Ag was coated as: 20.0 µg, 17.5 µg, 15 µg, and 10.0 µg 15-DON-OVA per well for rounds 1, 2, 3, and 4, respectively. Two replicates of target Ag were used in each round of panning. Blank wells (one replicate) were maintained during panning by coating wells with PBS. Antigen and control wells for all panning rounds were coated with 100 µL·well\(^{-1}\) and incubated overnight at 4°C. Blocking buffer (2% w/v OVA in PBS) was used for panning rounds 1 and 2. To limit non-specific binding for rounds 3 and 4, blocking buffer was switched to casein (1% w/v in PBS). As well, phage from each round of panning were also pre-incubated with the respective blocking solution (100 µL) for 1 hour at 37°C before 100 µL of phage were added to 15-DON-OVA and PBS coated wells. After incubation of phage for 2 hours at 37°C, antigen and control wells were washed with PBST. Stringency of washing was increased by washing 5, 6, 7, and 8 times with 200 µL PBST for rounds 1, 2, 3, and 4 of panning, respectively.
Bound phage were eluted by adding 100 µL of freshly prepared triethylamine solution (35 µL TEA + 2.50 mL sterile H²O). Eluant was neutralized after incubation for 10 minutes by adding 200 µL of 1.0 M Tris HCl. Eluted phages after each round of panning were used to infect 2 mL E. coli TG1 cells for subsequent amplification as described in (Ghahroudi et al., 1997). Briefly, infected cells were incubated at 37°C for 30 min without shaking. A 2 µL aliquot was taken for phage titration, the remaining culture volume was transferred to 6 mL 2xYT medium + ampicillin (100 µg·mL⁻¹) followed by incubation and shaking (37°C, 30-60 min). The culture was subsequently superinfected with 900 µL of helper phage M13 K07 (1 x 10¹¹ cfu·mL⁻¹) (New England Biolabs) followed by incubation with no shaking (37°C, 15 min.) and incubation with shaking (37°C, 30-60 min). Cells were transferred to 92 ml 2xYT medium containing ampicillin and kanamycin (final concentrations of 100 mg·mL⁻¹ and 50 µg·mL⁻¹, respectively). After amplification of eluted phages overnight (37°C with shaking), phage titers were estimated by plating infected cells on 2xYT + ampicillin 100 mg·mL⁻¹ agar plates. Input and eluted phage were quantified by serial dilution with PBS and plating on LB-AMP (data not shown). The number of colonies in each plate were counted and compared with the colony numbers from subsequent rounds of panning.

4.3.7 Phage ELISA

Individual colonies obtained after four rounds of panning were tested against 15-DON-OVA conjugate in a phage ELISA according standard procedures (Ghahroudi et al., 1997). Briefly, clones were grown in 2xYT medium + ampicillin (100 mg·mL⁻¹) + 0.1 % glucose medium to OD₆₀₀ = 0.3 – 0.5, and infected with M13K07 helper phage (37°C no
shaking, 30 min) followed by kanamycin (50 μg·mL⁻¹) and amplification overnight (37°C with shaking). Cultures were centrifuged (5,000 rpm, 20 min., 4°C) to pellet the cells, followed by the addition of 100 μL of supernatant (recombinant phage particles) to pre-coated microtiter plate wells. Treatments were two replicates of 15-DON-OVA (0.5 μg·well⁻¹ in PBS or 0.15 M carbonate-bicarbonate buffer). Blank wells were OVA (2.0 μg·well⁻¹ PBS) and BSA (2.0 μg·well⁻¹ PBS). Negative control was 100 μg·well⁻¹ of M13K07 in PBS. After 2-hour incubation at 37°C, microtiter plate wells were washed five times with PBST followed by addition of anti-M13 HRP conjugate (1:5000). V₃H₃-phages bound to DON were detected by the addition of 100 μL of HRP substrate (KDL) followed by colourimetric development and assessment as previously described.

4.3.8 Colony PCR and Sequencing

Universal primers M13R and PN2 were used to amplify the V₃H₃ fragments of the positive clones determined by phage ELISA. Sequencing was carried out using the ABI PRISM™ Big Dye® Terminator Cycle Sequencing Reaction Kit (Perkin-Elmer, Wellesley, MA) on a 373 DNA sequencer apparatus (Perkin-Elmer). Sequencing data were aligned and numbered according to the Kabat numbering system (Kabat et al., 1991). The unique nucleotide sequence for the dominant clone (repeated in 38 of 100 clones) that bound to 15-DON-OVA and not to OVA control was termed “NAT-267”.

4.3.9 Monomer Expression and Purification

Restriction enzyme sites BbsI and BamHI were added to the 5’ and 3’ ends of the NAT-267 V₃H₃ DNA fragment using a PCR involving gene-specific sense primer VHH-BbsI (5’-TATGAAGACACCAGGCCAGGTGCAGCTGGTGGAGTCT-3’) and anti-
sense primer VHH-BamHI (5’-CGCGGGATCCTGAGGAGACGTTGACCTGGGT-3’). The amplified DNA was then digested with BbsI and BamHI restriction enzymes and ligated into digested pSJF2 vector using standard techniques (Tanha et al., 2003). Competent E. coli TG1 cells were transformed and clones expressing DON-specific recombinant \(V_{HH}\) were grown in 1-liter cultures of 2xYT medium + ampicillin (100 mg·mL\(^{-1}\)) with 0.1% glucose to an OD\(_{600}\) of 0.8. Cultures were induced with 0.5 mM IPTG and grown overnight on a rotary shaker at 28°C. After confirmation of expression by SDS-PAGE and Western blotting, recombinant mNAT-267 \(V_{HH}\) protein was extracted from the bacterial periplasm by standard methods (Anand et al., 1991) and purified by immobilized metal affinity chromatography (IMAC) and quantified as described elsewhere (MacKenzie et al., 1994). The state of aggregation of the purified protein was checked by size exclusion chromatography on Superdex 200 (Amersham Biosciences).

### 4.3.10 Pentamerization of \(V_{HH}\) Monomer

Restriction enzyme sites BbsI and ApaI were added to the 5’ and 3’ ends of the NAT-267 \(V_{HH}\) DNA fragment using a PCR involving the gene-specific sense primer VHH-BbsI and antisense primer VHH-ApaI (5’-ATTATTATGGCCCTGAGGAGACG GTGACCTGGGT-3’). Amplified NAT-267 DNA was digested with BbsI and ApaI restriction enzymes and ligated into digested pVT2 using techniques as described in (Zhang et al., 2004). A \(V_{HH}\) pentamer of the dominant clone was formed by fusion of monomer DNA to the verotoxin B subunit encoded within the pVT2 expression vector. pNAT-267 DNA was then used to transform E. coli TG1 according to standard techniques.
4.3.11 Pentamer Expression and Purification

The same conditions which were used to express the mNAT-267 VdH protein, were applied for expression of pNAT-267 VdH. In accordance with standard protocols (Zhang et al., 2004), expressed proteins were extracted by whole-cell lysis. Briefly, cells were pelleted by centrifugation (20 min, 6,000 xg, 4°C) and resuspended in 25 mL of ice-cold lysis buffer (10 mM Hepes, 500 mM NaCl; 20 mM Imidazole; pH 7.4) followed by centrifugation (1 hour, 6,000xg, 4°C). Supernatants were filtered (0.22 µm Millipore filter) and protein extracts were centrifuged again (1 hour, 6,000 xg, 4°C). After confirmation of protein expression by SDS-PAGE and Western Blotting, pentabodies were purified by IMAC as described above. The state of aggregation was checked by size exclusion chromatography on Superdex 200 (Amersham Biosciences).

4.3.12 Determination of Binding Affinity

4.3.12.1 Fluorescence Polarization Assay

DON-fluorescein tracer was prepared according to standard methods (Maragos et al., 2002). Briefly, DON (0.625 mg in 100 µL acetone) was added to 1,1-carbonyl-diimidazole (2 mg) and allowed to mix (2 hours, 24°C). 6-aminofluorescein (10 mg) was dissolved in 1 mL sodium carbonate (1 mL, 0.1 M). 6-aminofluorescein (100 µL) was added to DON-1,1-carbonyl-diimidazole, final volume 200 µL. After reaction for 48 hours at 2 - 8°C, DON–fluorescein solution (20 µL; approx. 20 mM) was spotted onto LK6F silica gel plates (1,000 µm Preparative layer thickness 20 x 20 cm; Whatman) along with samples of DON (20 mM), DON-1,1-carbonyl-diimidazole (20 mM), and 6-aminofluorescein (20 mM), as reference standards. Thin layer chromatography (TLC)
plates were developed in chloroform:methanol:acetic acid (90:10:1 v/v/v). Each product visualized at 366 nm was scraped from the plate into a vial containing 0.2 mL methanol (2 - 8°C) incubated over-night and stored at -20°C until required.

Fluorescence polarization was measured with a Perkin Elmer Envision 2100 multilabel reader. To determine the operating dilution of fluorescent tracer within the assay, DON-fluorescein was serially diluted 1:2 in 1 mg∙mL⁻¹ BSA from 1:100 to 1:102,400 and analyzed by fluorescence polarization (100 μL-well⁻¹) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The operating dilution of DON-fluorescein conjugate was the dilution yielding a total fluorescence within the linear range of the above titration, nearest the lower plateau to represent the lowest accurately-measurable concentration of DON-fluorescein conjugate, thus requiring the least amount of V₁H to saturate the system. To determine the dilution of mNAT-267 and pNAT-267 V₁H fragments, DON-fluorescein conjugate was diluted in 1 mg∙mL⁻¹ BSA and mixed with serial dilutions of V₁H in PBS. These mixtures were allowed to react (10 min., 22°C) in the dark and followed by fluorescence polarization (100 μL-well⁻¹) analysis with excitation and emission wavelengths of 488 nm and 530 nm, respectively.

A standard curve of competition between DON-fluorescein (1:8,000 dilution in PBS) and free DON in solution at concentrations ranging from 5 μg∙mL⁻¹ to 9.8 ng∙mL⁻¹ was prepared using a competition assay. V₁H antibody fragments were diluted 1:50 (v/v) in PBS containing DON serially diluted 1:2 (v/v) from an initial concentration of 10 μL∙mL⁻¹ (six replicates for each concentration). Mixtures were allowed to react for 1 hour at 22°C after which they were applied (80 μL-well⁻¹) to a black 96-well microtitre
plate. DON-fluorescein was diluted 1:640 (v/v) in 1 mg·mL\(^{-1}\) BSA and added (20 µL-well\(^{-1}\)) to the serum-DON solution in each well. Mixtures reacted for 10 minutes at 22°C in the dark and were analyzed by FPA. Cross reactivity with other trichothecene mycotoxins (i.e., 3-AcDON, 15-AcDON, DAS, T2, and NEO) was determined by substitution of DON with each mycotoxin within the serial dilutions as previously noted.

### 4.3.12.2 Surface Plasmon Resonance

The interaction of mNAT-267 V\(_{H}\)H and immobilized 15-DON-HRP conjugate was determined by SPR using the BIACORE 3000 biosensor system (Biacore, Inc., Piscataway, NJ). Data were analyzed with BIAevaluation 4.1 software. Approximately 500 resonance units (RUs) of 15-DON-HRP conjugate and 190 RUs HRP (control) were immobilized on a CM5 research grade sensorchip (BIACORE), respectively. Immobilizations were carried out at protein concentrations of 50 µg·mL\(^{-1}\) for conjugate and 200 µg·mL\(^{-1}\) for HRP in 10 mM acetate (pH 4.0) using an amine coupling kit supplied by the manufacturer. Before analysis, the mNAT-267 V\(_{H}\)H was passed through a Superdex 75 (GE Healthcare) column. mNAT-267 V\(_{H}\)H was injected over HRP reference and 15-DON-HRP surfaces at concentrations of: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 µM for 6 sec. All analyses were carried out at 25°C in 10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20 at a flow rate of 100 µL·min\(^{-1}\) with 6-sec pulse injection of mNAT-267 V\(_{H}\)H followed by 6-sec. injection of 10 mM sodium borate, pH 8.5, in 1 M NaCl (BIACORE) for regeneration. Steady state binding (Req) at each concentration of V\(_{H}\)H monomer was determined for K\(_D\) analysis.
To evaluate competitive inhibition of mNAT-267 to free DON, 15-DON-HRP conjugate and 190 RUs of HRP (control) were immobilized on a CM5 research grade sensorchip (BIACORE) as noted above. All analyses were conducted at 25°C in 10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20 at a 100 μL·min⁻¹ flow rate with 6 sec pulse injection of Vₜₚ monomer in absence or presence of free DON (50 and 500 μM).

pNAT-267 Vₜₚ and control pentabody ‘C3C-BSA8’ were captured on a Ni²⁺ charged sensorchip NTA (BIACORE) at densities of 10,000 RUs and 6,000 RUs, respectively. DON toxin was injected over both surfaces at a flow rate of 20 μg·min⁻¹ at concentrations ranging from 0.1 - 2 mM. Pentamers were regenerated with 1-min injection of 350 mM EDTA in 10 mM HEPES pH 8.5 containing 150 mM NaCl and 0.005% P20 surfactant. All analyses were conducted at 25°C in 10 mM HEPES, pH 7.4 containing 150 mM NaCl and 0.005% surfactant P20.

4.3.12.3 Competitive Inhibition ELISA

Microtiter plates were coated with either 15- DON-OVA conjugate (0.5 μg·well⁻¹, 0.15 M bicarbonate buffer) or OVA (2.0 μg·well⁻¹, PBS) and blocked with 1.0% casein (PBS) (2 hours, 37°C). Eleven serial dilutions of DON ranging from 0.005 to 10.00 μg·mL⁻¹ (17 nM to 34 μM, respectively) were prepared in PBS. Purified soluble mNAT-267 or pNAT-267 Vₜₚ (100μL) were added in equal volumes to each dilution series for a final DON concentration ranging from 0.0025 to 5 μg·mL⁻¹ (or 8.4 nM to 17 μM, respectively). After vortexing and incubation (2 hours, 37°C), DON–Vₜₚ solutions were added to the ELISA-plate wells and incubated (1 hour, 37°C) followed by five washes
with PBST. Rabbit anti-6 His-HRP conjugate (100 µL; 1:5,000 in PBS) was added to each well followed by incubation (1 hour, 25°C). HRP substrate (KPL, Gaithersburg, MD) was added followed by the addition of 1 M H₃PO₄. Absorbance at (450) nm was measured with an ELISA plate reader. Cross reactivity with other trichothecene mycotoxins (i.e., 3-AcDON, 15-AcDON, DAS, T2, and NEO) was tested by substitution of DON-standards with each mycotoxin using the serial dilutions previously noted.

4.4 Results

4.4.1 Polyclonal Response

4.4.1.1 Direct ELISA

ELISAs using polyclonal llama sera sampled either 42 or 70 days after initial immunization with 15-DON-BSA + TiterMax Adjuvant confirmed a robust humoral response over a dilution series ranging from 1:1 to 1:2,097,152 (Figure 4.1). Polyclonal titer, expressed as 50% of maximum absorbance for pre-immune, 42, and 70 day sera were estimated to be 1:250; 1:70,800; and 1: 56,200, respectively. Overall response was regarded as mycotoxin-specific as the response of polyclonal sera to control antigen (2% OVA) was markedly less than to wells coated with 15-DON-OVA (Figure 4.1).
Figure 4.1  ELISA of polyclonal llama serum response to 250 μg 15-DON-BSA conjugate. 42-and 70-day serum samples each taken one week after third and fifth immunizations, respectively. X-axis shows dilutions from 1 to 1:2,097,152 in PBS. Antigen wells were coated with 15-DON-OVA (0.5 μg·well⁻¹). Control wells were coated with OVA (2.0 μg·well⁻¹).

4.4.1.2 Fluorescence Polarization

TLC of DON-fluorescein conjugate yielded three distinct bands (R₇ = 0.29, 0.24 and 0.18). Llama anti-DON serum had affinity for each conjugate using FPA (data not shown). DON-fluorescein conjugate corresponding to R₇ = 0.29 was used for the development of an optimized FP assay (FPA) since it was the most plentiful product and had the most consistent results.

A competitive polyclonal FPA was established with 56-day polyclonal serum based on a plot of polarization/maximum polarization observed (i.e. P/P₀) against DON concentration. The linear working range of the FPA was 10 – 5,000 ng·mL⁻¹ with limits of detection and quantitation of 19 ng·mL⁻¹ and 50 ng·mL⁻¹, respectively (data not shown). The IC₅₀ from the competitive FPA to DON was 229 ng·mL⁻¹, a value which is
significantly higher than previously published values based on similar polyclonal assays to assess hapten binding (e.g., Furzer et al., 2006; Maragos et al., 2002).

4.4.2 HCAb and cIgG Response

4.4.2.1 Protein A and G Chromatography

Fractionation of pre-immune, 42- and 70-day serum samples confirmed an increase in HCAb and cIgG content after immunization with 15-DON-BSA. A greater proportion of HCAb relative to cIgG was noted within fractionated pre-immune serum, while immunization with 15-DON-BSA increased the relative proportion of cIgG in the 42- and 70-day hyper-immunized sera (Table 4.2). Absolute and relative quantities of HCAb and cIgG fractions were in agreement with published values (Muyldermans, 2001; van der Linden et al., 2000).

<table>
<thead>
<tr>
<th>IgG</th>
<th>G1 (HCAb)</th>
<th>G2 (cIgG)</th>
<th>A1 (HCAb)</th>
<th>A2 (HCAb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre 42d 70d</td>
<td>Pre 42d 70d</td>
<td>Pre 42d 70d</td>
<td>Pre 42d 70d</td>
</tr>
<tr>
<td>Vol. (mL)</td>
<td>2.3 2.5 2.8</td>
<td>6.0 6.4 8.5</td>
<td>1.8 1.9 2.1</td>
<td>2.0 1.8 1.7</td>
</tr>
<tr>
<td>Ratio* (% v/v)</td>
<td>19.0 19.8 18.3</td>
<td>49.6 50.8 56.4</td>
<td>14.9 15.1 14.0</td>
<td>16.5 14.3 11.3</td>
</tr>
<tr>
<td>Conc. (mg·mL⁻¹)</td>
<td>0.11 0.13 0.05</td>
<td>0.71 0.21 0.10</td>
<td>0.10 0.14 0.16</td>
<td>0.12 0.16 0.16</td>
</tr>
<tr>
<td>Amount (mg)**</td>
<td>0.25 0.43 0.36</td>
<td>0.30 4.54 1.79</td>
<td>0.18 0.19 0.29</td>
<td>0.32 0.22 0.27</td>
</tr>
</tbody>
</table>

* % v/v ratio calculated as volume of fraction ÷ total volume.
** Amount = Conc. (mg·mL⁻¹) x Vol. (mL).

4.4.2.2 ELISA on Polyclonal Serum Fractions

Relative to pre-immune fractions, both cIgG and HCAb fractions from 42- and 70-day sera had significantly greater binding to 15-DON-OVA than to OVA. The cIgG
fractions of 42- and 70-day serum samples had the greatest overall binding to 15-DON-OVA. However, HCAb fractions also showed significant binding to 15-DON-OVA (data not shown). Based on the positive HCAb ELISA results, a recombinant V_{H} phage-display library was constructed using HCAb cDNA from both the 42- and 70-day sera.

### 4.4.3 Library Construction

The initial step in V_{H} library construction was PCR amplification of a mixture of 42- and 70-day variable heavy chain leukocyte DNA. Sense primer mixture “MJ1.2.3 Back” and hinge-specific anti-sense primer mixture “CH_{2}b_{3} + CH_{2}” (Table 4.1) annealed to conserved regions of cIgG and HCAb DNA to produce PCR fragments of 620 – 690 bp and 900 bp, respectively. VH/VHH–specific primers MJ7 and MJ8 (Table 4.1) amplified 620 – 690 bp HCAb DNA and to add SfiI restriction sites to produce 400 – 450 bp PCR products which were gel purified and ligated into pMED1 phagemid. Library diversity was determined by sequencing of 30 recombinant clones and by concurrent digestion of PCR-amplified V_{H} fragments from individual colonies with 4-mer BstN1 restriction endonuclease. Library size was determined to be approx. 10⁹ primary recombinants.

### 4.4.4 Panning and Phage ELISA

The recombinant phage-displayed V_{H} library was panned against 15-DON-OVA and OVA (control) through four rounds of panning. Favorable ratios of input and output phage specific to the target mycotoxin were maintained through a continuous reduction in 15-DON-OVA conjugate coating for each round of panning and a switch in blocking agents from OVA to casein between panning rounds two and three.
Phage ELISA conducted on ten randomly selected clones from the fourth round of panning revealed three clones (phage # 2, 6, and 7) that bound to 15-DON-OVA, but not to control proteins (i.e., OVA, BSA). Further phage ELISA screening of 100 randomly selected output clones from the fourth round of panning produced 38 clones that bound to 15-DON-OVA, but not to control proteins, which suggested that ~ 40% of the phage clones were hapten-specific (data not shown).

4.4.5 V_H Sequence Results

Nucleotide sequencing of positive phage ELISA clones revealed a single dominant nucleotide sequence (‘NAT-267’) encoding a V_H which bound to 15-DON-OVA, but not protein controls. Relative to previously published V_H sequences, the NAT-267 sequence was unique (Figure 4.2).

<table>
<thead>
<tr>
<th>FR-1 (1-25)</th>
<th>CDR-1 (26-35)</th>
<th>FR-2 (36-49)</th>
<th>CDR-2 (50-65)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 10 20 30 40 50 60</td>
<td>... ... ... ...</td>
<td>... ... ... ...</td>
<td>... ... ... ...</td>
</tr>
</tbody>
</table>

QVQLEESGGGLVQAGGSLRLSCAAS GRTSSNTFVG WFRQAPGKERE FVA AIRSDDRTYYAASVRG

<table>
<thead>
<tr>
<th>FR-3 (66-94)</th>
<th>CDR-3 (95-102)</th>
<th>FR-4 (103-113)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 80 90 100 110</td>
<td>... ... ... ...</td>
<td>... ... ... ...</td>
</tr>
</tbody>
</table>

RFTISGDSDKVVALQMSSLRPEDTA VYCCA TRTWLVTGQSDYPY WKGQTVVSS

Figure 4.2 Amino acid sequence of NAT-267 V_H from fourth round of panning. Recombinant V_H CDR (underlined) and FR delimitations as well as amino acid insertions (52a, 82a,b,c, 100a,b,c,d,e,f) defined according to Kabat numbering scheme (Kabat et al., 1991).

Although the sequence of the Framework Region (FR) was similar to that of the hapten binder ‘RR6-R2’ (Spinelli et al., 2001), residues within the three complementarity determining regions (CDR) were novel. As commonly observed for V_H hapten binders,
NAT-267 did not contain cysteine residues within CDR 1 or 3, which is commonly believed to serve as the basis of an interloop disulfide bridge within recombinant VH protein structure (Figure 4.2). Except for clones that bound only to control proteins, all VH sequences isolated had relatively long predicted CDR-3 regions (12 – 18 amino acids) (data not shown).

4.4.6 Expression of Soluble VH Protein

Recombinant protein fractions of approximately 16 kDa in size, containing the mNAT-267 VH were pooled. From the absorption measurement at 280 nm, a yield of 3 to 6 μg of purified protein per liter of E. coli culture was calculated. The purified protein was concentrated, by ultrafiltration, to 1.3 mg·mL⁻¹ in PBS; no aggregation was observed.

Purification of pNAT-267 VH of approximately 130 kDa in size was successful using standard IMAC procedures. Aggregated pentabodies were removed by size exclusion chromatography on Superdex 200 column. Overall yield of pNAT-267 was determined to be 3 to 9 μg·L⁻¹ of E. coli culture. Ultrafiltration was used to obtain a final pentamer concentration of 1.0 mg·mL⁻¹ in PBS; no aggregation was observed.

4.4.7 VH Affinity Measurements

4.4.7.1 Fluorescence Polarization

Initial FPA analysis based on step-wise titrations of both mNAT-267 and pNAT-267 confirmed identical binding of both recombinant proteins to DON-fluorescein tracer diluted 1:8,000 in skim milk (1 mg·mL⁻¹) (data not shown). Plots, based on \( P/P_0 \) versus concentration of free hapten, were used to determine \( IC_{50} \) values, or concentration required for 50% inhibition of binding. Competitive FP inhibition assays determined an
IC\textsubscript{50} value of 0.82 μM for free DON versus 56-day polyclonal llama serum (Figure 4.3A). Neither the monomer nor pentamer bound to soluble DON. Likewise, FP inhibition with 3-AcDON determined an IC\textsubscript{50} value of 0.62 μM for 56-day polyclonal llama serum, but no binding for NAT-267 monomer or pentamer (Figure 4.3B). FP inhibition with 15-AcDON determined an IC\textsubscript{50} value of 1.42 μM for 56-day polyclonal serum, and IC\textsubscript{50} values of 1.24 and 0.50 μM for NAT-267 monomer and pentamer V\textsubscript{H}H, respectively (Figure 4.3C). FP inhibition assays with diacetoxyscirpenol (DAS), neosolaniol (NEO), and T-2 trichothecene toxins indicated no specific binding to polyclonal serum, mNAT-267 or pNAT-267 V\textsubscript{H}H (data not shown).

4.4.7.2 Surface Plasmon Resonance

Binding kinetics of mNAT-267 V\textsubscript{H}H binding to 15-DON-HRP were determined by SPR (BiaCore™) analysis. Monomer was injected over HRP control and 15-DON-HRP bound to a SPR sensor chips at concentrations of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μM to generate an overlay sensogram of mNAT-267 binding to 15-DON-HRP conjugate (Figure 4.4). Trace amounts of aggregate mNAT-267 V\textsubscript{H}H contamination were likely responsible for second phase binding to 15-DON-HRP conjugate, which could be seen on sensorgram traces as trends with slightly positive slope. To minimize this effect, data were collected at the highest flow rate (100 μL·min\textsuperscript{-1}), and binding kinetics were measured as soon as first phase binding reached steady state, or ‘Req’ (Figure 4.4).
Figure 4.3  FP inhibition of mNAT-267 and pNAT-267 V_{H}H binding and 56-day polyclonal llama serum binding to DON-fluorescein tracer with DON toxin (A); 3-AcDON (B); and 15-AcDON (C) from 5 μg·mL\(^{-1}\) to 9.8 ng·mL\(^{-1}\). IC\(_{50}\) of llama serum binding to: DON; 3-AcDON; and 15-AcDON = 0.82 μM; 0.62 μM; and 1.42 μM, respectively. IC\(_{50}\) for mNAT-267 and pNAT-267 binding to 15-AcDON = 1.24 μM and 0.50 μM, respectively. For each assay, error bars represent ±1 SD, number of replicates = 6. DON-fluorescein tracer within each assay diluted 1:8,000 in PBS.
Figure 4.4   Overlay sensorgrams of mNAT-267 $V_{hH}$ steady state (Req) binding to 15-DON-HRP conjugate. mNAT-267 $V_{hH}$ injected over HRP reference and 15-DON-HRP surfaces at the concentrations of 1 (lowest trace), 2, 3, 4, 5, 6, 7, 8, 9 and 10 (highest trace) μM for 6 seconds.

An assessment of steady state binding (Req) relative to concentration of mNAT-267 $V_{hH}$ confirmed first-order binding kinetics (Figure 4.5A). A Scatchard plot of Req binding at each mNAT-267 $V_{hH}$ concentration relative to actual binding demonstrated binding to immobilized 15-DON-HRP with a dissociation constant ($K_D$) of 5 μM (Figure 4.5B). Independent association and dissociation rates were too fast to calculate.

Free DON (50 and 500 μM) was mixed with mNAT-267 $V_{hH}$ (5 μM) to test competitive inhibition of monomer binding to bound 15-DON-HRP. No competitive inhibition was observed indicating that mNAT-267 had no affinity for soluble DON. This observation was further confirmed by SPR analysis of the interaction between pNAT-267 $V_{hH}$ and soluble DON. After capture of pNAT-267 to the SPR sensor chip, no binding interaction was noted between the pentamer and concentrations of soluble DON ranging from 0.1 to 2 mM (data not shown).
Figure 4.5  (A) Steady state (Req) affinity binding (RU) mNAT-267 V<sub>H</sub>H to 15-DON-HRP conjugate for ten monomer concentrations, as shown in Figure 4.4. (B) Scatchard plot of steady state ‘Req’ binding (RU) relative to Req / C (mNAT-267 V<sub>H</sub>H concentration) (RU·μM<sup>-1</sup>). Dissociation constant (K<sub>D</sub>) = 5 μM.

SPR analysis was not used to assess interaction of mNAT-267 or pNAT-267 to 3-AcDON and 15-AcDON. Unfortunately, it was not possible to prepare functional solutions since the relative water solubilities of these acetylated toxins were much lower than DON. Furthermore, it was impractical to dissolve the acetylated toxins in organic solvent (e.g., acetone) followed by dilution in PBS since the solvent adversely affects SPR measurement relative to control assessments.
4.4.7.3 CI ELISA

Checkerboard immunoassay titrations confirmed optimum binding of NAT-267 $V_{1\text{H}}$H (relative to OVA controls) based on dilution of monomer and pentamer at 0.38 $\mu$g∙mL$^{-1}$ within ELISA wells coated with 15-DON-OVA at 0.5 $\mu$g·well$^{-1}$. Although CD ELISA consistently showed that 15-AcDON inhibited mNAT-267 and pNAT-267 binding to 15-DON-OVA, it was not possible to precisely define the respective IC$^{50}$ values below 10 $\mu$M (data not shown). Binding of monomer and pentamer to 15-DON-OVA coated plates was not affected by competition with serial dilutions of DON or 3-AcDON at concentrations ranging from 8.4 nM (0.0025 $\mu$g∙mL$^{-1}$) to 17 $\mu$M (5.0 $\mu$g∙mL$^{-1}$).

4.5 Discussion

DON toxin and its acetylated metabolites are low molecular weight (296 to 338 Da) highly stable trichothecene mycotoxin contaminants of maize and small grain cereals (Desjardins, 2006). Trichothecene mycotoxins, a group of over 148 related compounds, are known to cause severe toxicosis in humans and farm animals. For example, swine vomit and display a wide variety of other symptoms of toxicosis including immunosuppression, after consumption of contaminated feed (Canady et al., 2001). Most countries have legislation and strict guidelines regarding acceptable levels of mycotoxins in contaminated cereals (Rotter et al., 1996).

Immunoassay techniques that can detect and quantify DON and structurally-similar mycotoxins use polyclonal serum and monoclonal antibodies. These assays provide highly accurate mycotoxin measurement. However, the development of toxin-specific immunoglobulins is difficult since it typically involves the repeated immunization of
animals and the use of highly specialized, laborious techniques to produce specific polyclonal sera or mAbs with high specificities and affinities.

The development of toxin-specific recombinant antibody (rAb) fragments such as single-chain variable fragment (scFv) and $V_{H}$H provides an alternative means of producing low-cost and abundant quantities of high affinity binders within common recombinant expression hosts. Although scFv fragments often display binding affinities equivalent to the parent mAb (e.g., Wang et al., 2007), in practical terms, difficulties in obtaining purified protein, and challenges associated with protein degradation and the formation of insoluble aggregates and inclusion bodies within bacterial cultures (Choi et al., 2004), has limited large-scale application of this technology (Arbabi-Ghahroudi et al., 2005). We pursued the development of DON-specific single domain $V_{H}$H fragments as an alternative to the conventional IgG mAb and scFv rAb formats, as $V_{H}$H are smaller and generally have favorable expression from microbial cells.

This study describes the successful isolation and cloning of a single-domain $V_{H}$H gene from a hyperimmunized llama library, followed by soluble expression as a monomeric and pentameric $V_{H}$H protein, and characterization of binding kinetics to various DON toxins and their respective protein conjugates. To our knowledge, this is the first example of the development of a single-domain $V_{H}$H binder to a mycotoxin.

Before $V_{H}$H library construction and panning, we confirmed a robust and toxin-specific response to immunization with 15-DON-BSA within peripheral leukocytes (Figure 4.1). Serial dilution of whole and cIgG and HCAb fractionated serum tested by ELISA confirmed a polyclonal response specific for 15-DON-OVA. An initial
competitive FP inhibition assay confirmed that whole llama serum had affinity for free DON toxin ($IC_{50} = 229 \text{ ng}\cdot\text{mL}^{-1}$). Based on these results, we concluded that the methods and reagents used to generate a polyclonal response were optimized. Although the fractionated llama serum demonstrated a greater increase in cIgG content relative to HCAb fractions (Table 4.2), we concluded that the HCAb response was pronounced enough to warrant construction of a hyper-immunized $V_{H}H$ library.

Peripheral leukocytes were used as sources of mRNA for the cloning of $V_{H}H$ coding sequences to construct the hyperimmunized library (reviewed in Muyldermans, 2001). Variable regions of HCAb and IgG immunoglobulins were amplified by nested PCR based on hinge-specific primers. $V_{H}H$-specific PCR products were excised from an agarose gel and re-amplified with hinge-specific primers. The latter primers added the required $SfiI$ restriction endonuclease enzyme site onto amplified $V_{H}H$ DNA to enable digestion and ligation into the pMED1 phagemid system. Results based on colony PCR, DNA fingerprinting, and successive plate dilutions of transformed TG1 $E. coli$ showed the $V_{H}H$ library to be diverse and of sufficient size to proceed with biopanning.

Four rounds of panning against decreasing concentrations of 15-DON-OVA coupled with increased washing stringency yielded a single dominant $V_{H}H$ clone (termed NAT 267) at a frequency of 38%. Subsequent phage ELISA confirmed that NAT-267 bound to 15-DON-OVA with little or no binding to OVA (control). The panning process was deemed successful despite the fact that it was not based on a process of affinity selection through elution with free DON hapten which may have improved the selection of high affinity toxin-specific $V_{H}H$ binders (Sheedy et al., 2007).
The NAT-267 V_H gene was well expressed in monomeric and pentameric forms within \textit{E. coli}. The relatively high concentrations of expressed V_H are comparable to the values published for scFv and Fab (Maynard and Georgiou, 2000). The high yield of monomer and pentamer forms of NAT-267 was attributed to the soluble and inherently robust nature of recombinant V_H proteins (Ghahroudi et al., 1997; Goldman et al., 2006; Ladenson et al., 2006). Direct ELISA assays confirmed that soluble V_H monomer and pentamer bound to 15-DON-OVA, but not to OVA (control). Given identical microtiter plate dilution regimes, the ELISA signal for the pentamer was consistently higher than for equivalent monomer concentrations. This result suggested that the dominant V_H clone was selected based on avidity of a multivalent phage during panning to hapten-protein conjugate rather than affinity to free hapten, which is a limitation when panning with multivalent Ab-displaying phage.

A series of assays to determine V_H monomer and pentamer binding specificity confirmed that purified NAT-267 monomer and pentamer fragments recognized 15-AcDON (Figure 4.3C) but not DON (Figure 4.3A), 3-AcDON (Figure 4.3B), or the other trichothecene toxins tested (diacetoxyseirpenol, neosolaniol, and T-2 toxin).

The free hydroxyl group at position 15 of DON and 3-AcDON may have interfered with NAT-267 V_H binding. Conversely, the presence of an acetyl ester group (≈ 42 Da) at position 15 of the trichothecene structure (Figure 4.6) may have altered the molecular structure and conformation to impart V_H binding to 15-AcDON. Given that the DON toxin was conjugated to carrier proteins by a hemi-succinate linker at position 15 for both the antigen (15-DON-BSA) and the coating conjugate (15-DON-OVA) used for panning,
it was postulated that the V_{H}H paratope recognized an epitope at or in close proximity to position 15 of the DON molecule.

\[
\begin{align*}
&\text{Trichothecene} & \text{MW}^a & \text{Oxygenation and Esterification at Position}^b \\
&3\text{-Acetyl-DON} & 338 & \text{OAc} & \text{H} & \text{OH} & =\text{O} & \text{CH}_2\text{-OH} \\
&15\text{-Acetyl-DON} & 338 & \text{OH} & \text{H} & \text{OH} & =\text{O} & \text{CH}_2\text{-OAc} \\
&\text{Deoxynivalenol} & 296 & \text{OH} & \text{H} & \text{OH} & =\text{O} & \text{CH}_2\text{-OH} \\
&\text{Diacetoxyscirpenol} & 366 & \text{OH} & \text{OAc} & \text{H} & \text{H} & \text{CH}_2\text{-OAc} \\
&\text{Neosolaniol} & 382 & \text{OH} & \text{OAc} & \text{H} & \text{OH} & \text{CH}_2\text{-OAc} \\
&T-2\text{ Toxin} & 466 & \text{OH} & \text{OAc} & \text{H} & \text{OIsoval} & \text{CH}_2\text{-OAc} \\
\end{align*}
\]

$^a\text{MW} = \text{Molecular Weight}$

$^b\text{OAc} = \text{acetyl ester. OIsoval = isovalerate ester.}$

**Figure 4.6** Structure, composition, and molecular weight of trichothecene mycotoxins used in this study (Desjardins, 2006).

Highly-specific V_{H}H binding to 15-AcDON was therefore regarded as a function of both the linker used to fix DON to carrier proteins and the panning methods, while the physico-chemical properties of these ligands, such as water solubility and molecular size, may have had little impact on V_{H}H specificity. It would be interesting to test this hypothesis by panning the V_{H}H library against a DON-protein conjugate with different linker chemistry and/or a different conjugation position (e.g., 3-DON-OVA).
Based on competitive FP assays, IC$_{50}$ values determined by competition with 15-AcDON were 1.24 and 0.50 μM for the V$_{H}$H monomer and pentamer, respectively. These values are comparable to IC$_{50}$ of 1.42 μM for 15-AcDON using 56-day polyclonal llama serum in a competitive FP assay (Figure 4.3C). Unfortunately, it was not possible to validate FP results by CD ELISA since 15-AcDON inhibition values for both the monomer and pentamer could not be precisely determined. The inherent difficulty in establishing consistent IC$_{50}$ values by CD ELISA was attributed to the relatively fast dissociation rate of both the NAT-267 monomer and pentamer coupled with the continuous removal of V$_{H}$H from the non-equilibrium binding system by successive washing steps after initial binding. Furthermore, the limited solubility of 15-AcDON prevented SPR analysis of its binding to chip-immobilized NAT-267 V$_{H}$H. However, SPR analysis of NAT-267 monomer binding to chip-immobilized 15-DON-HRP yielded a dissociation constant ($K_d$) of 5 μM, which is similar to the IC$_{50}$ determined in the competitive FP assay using NAT-267 (1.24 μM).

Unlike reports associated with poor scFv expression and isolation (Choi et al., 2004), our monomeric and pentameric V$_{H}$H fragments were found to be well expressed, stable and not prone to aggregation or formation of insoluble inclusion bodies. Furthermore, unlike scFv for DON toxins, NAT-267 V$_{H}$H was highly specific to the 15-AcDON moiety with no cross-reactivity to 3-AcDON or DON. Such specificity could aid in toxin-specific detection as a compliment to existing mAb assays. For example, the mAb (IgG) developed by (Maragos and McCormick, 2000) was reported to be very specific for DON and 3-AcDON with little or no affinity for 15-AcDON. When used in
conjunction this mAb-based ELISA, NAT-267 V_H would allow quantification of all three toxins (i.e., DON, 3-AcDON, and 15-AcDON). Furthermore, the mAb-based assay developed by Sinha et al. (1995), which detects both 15-AcDON and DON (i.e., 15-AcDON + DON), could be used in conjunction with NAT-267 V_H to determine the exact concentration of 15-AcDON as well as DON.

We compared NAT-267 V_H in terms of affinity and specificity with the other ‘hapten-specific’ V_H fragments that have been isolated from both naïve (Sheedy et al., 2006; Yau et al., 2003b) and hyper-immunized llama libraries (Alvarez-Rueda et al., 2007; Frenken et al., 2000; Ladenson et al., 2006; Spinelli et al., 2000). For example, the indoleacetic acid (IAA)-binding V_H CSF2A, isolated from a naïve llama library, had a K_D of 20 µM for free IAA (Sheedy et al., 2006). Unlike NAT-267, CSF2A cross-reacted to structurally-similar analogues. Furthermore, a picloram-specific V_H, 3-ID2, isolated from a naïve ribosome-display llama library had an IC_{50} of 800 µM for the hapten picloram (Yau et al., 2003b). In both cases, these naïve-library-based V_Hs had much lower affinities than did NAT-267.

Unlike the hapten-specific V_Hs isolated from naïve libraries (Sheedy et al., 2006; Yau et al., 2003b), the V_H sequences isolated from hyper-immunized llama libraries (e.g., Alvarez-Rueda et al., 2007; Ladenson et al., 2006; Spinelli et al., 2000) were in agreement with our work in that each V_H isolated was found to be hapten-specific with minimum cross-reactivity to structurally-similar analogues and their IC_{50} values as determined by competition with free hapten were in the µM range. For example, a caffeine-specific V_H, VSA2, had an IC_{50} of ca. 154 µM with minimum cross-reactivity
to structurally-similar haptens (Ladenson et al., 2006). Similarly, seven methotrexate (MTX)-specific V\textsubscript{H}Hs isolated from a hyper-immunized llama library (Alvarez-Rueda et al., 2007) had nanomolar affinities ($K_D = 29 - 515$ nM) for MTX-BSA; however, we estimate the affinity for the free hapten, which was not determined by the authors, to range from 10 and 70 $\mu$M based on IC\textsubscript{50} values (Alvarez-Rueda et al., 2007). Finally, the $K_D$ for an azo dye (Reactive-Red-6; M.W. = 728 Da)-specific V\textsubscript{H}H, known as RR6-R2, was determined to be 22 nM. Although the affinity of this V\textsubscript{H}H is much higher than NAT-267, the unusually high affinity of RR6-RR2 was attributed to a co-ordination bond between the copper of the dye and a histidine moiety in the V\textsubscript{H}H, rather than to a normal V\textsubscript{H}H-hapten interaction (Spinelli et al., 2000). Therefore, based upon the results of these authors (Alvarez-Rueda et al., 2007; Ladenson et al., 2006; Sheedy et al., 2006; Spinelli et al., 2000; Yau et al., 2003b), NAT-267 has the lowest hapten-based IC\textsubscript{50} of any hapten-specific V\textsubscript{H}H yet discussed in the literature.

The four scaffold FR residues within the NAT-267 V\textsubscript{H}H sequence had over 80% similarity with the V\textsubscript{H}H gene sequences of CSF2A (Sheedy et al., 2006), VSA2 (Ladenson et al., 2006), and RR6-R2 (Spinelli et al., 2000). Based on nucleotide acid identity, NAT-267 V\textsubscript{H}H belongs to camelid V\textsubscript{H}H sub-family I (Harmsen et al., 2000). Furthermore, each of the three CDRs of ‘NAT-267’ has unique amino acid residues. Contrary to published V\textsubscript{H}H sequences which have been shown to bind to large protein antigens, the CDR3 of NAT-267 is not constrained by an inter-loop disulfide bond to CDR1 since no cysteine residues were present within either region. Therefore, other than for the disulfide bridge formed between FR Cys-22 and Cys-92 (Figure 4,2), it is unlikely
that other disulfide bonds are present within NAT-267. Based on these observations, we concluded that the CDR1 to CDR3 inter-loop disulfide bonds present within many published sequences of V_Hs are important for facilitating V_H binding to large antigenic proteins but not to low molecular weight haptens (i.e. < 600 Da). Therefore, we hypothesize that the highly-specific binding of NAT-267 V_H to the haptens 15-AcDON and DON-fluorescein as well as to the protein conjugates 15-DON-OVA and 15-DON-HRP is likely conveyed by novel sequences within the non-crosslinked CDR residues. Furthermore, since the V_H paratope is not regarded to be a flat surface (Spinelli et al., 1996), we further hypothesized that high affinity interaction of NAT-267 V_H with the two haptens and coating conjugates occurs within flexible grooves or cavities between the three CDR regions (Spinelli et al., 1996; 2000; 2001).

4.6 Conclusions

This research further confirms that hapten-specific recombinant V_H fragments can be selected from a hyper-immunized llama V_H library. Both monomer and pentamer forms of the NAT-267 dominant clone selected by four rounds of panning was specific to the mycotoxin 15-AcDON as determined by competitive binding assay, and there was no cross-reactivity to closely related moieties of DON toxin. Although the dominant clone was both novel and highly specific to an acetylated moiety of the target mycotoxin, the nucleotide sequences for all four framework regions were similar to previously-isolated hapten-specific V_H sequences. Further research to assess different panning strategies and various affinity maturation strategies as a means of improving cross reactivity and overall binding affinity are now being pursued. Furthermore, since 15-AcDON has been
shown to have a similar eukaryotic cytotoxicity as DON (Binder et al., 1997; Poppenberger et al., 2003), we are focusing on expression of NAT-267 V_{H}H in eukaryotic test systems such as yeast and *Arabidopsis thaliana* to determine whether the negative effects of the toxin may be reduced or eliminated.

### 4.7 Acknowledgements

The authors of this paper would like to thank Tomoko Hirama (NRCC, Ottawa, Canada) for conducting the SPR analysis and Hong Tong-Sevinc (NRCC, Ottawa, Canada) for expression and purification of the V_{H}H clones. This research was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada, the Canada Research Chairs Program, the National Research Council (NRC) of Canada, and the Ontario Ministry of Agriculture and Food (OMAF).
5 RESEARCH CHAPTER 2: INTRACELLULAR EXPRESSION OF A SINGLE-DOMAIN ANTIBODY REDUCES CYTOTOXICITY OF 15-ACETYL-DEOXYNIVALENOL IN YEAST.

5.1 Abstract

15-Acetyl-Deoxynivalenol (15-AcDON) is a low molecular weight sesquiterpenoid trichotheocene mycotoxin associated with *Fusarium* Ear Rot of maize and *Fusarium* Head Blight of small grain cereals. The accumulation of mycotoxins such as deoxynivalenol (DON) and 15-AcDON within harvested grain is subject to stringent regulation as both toxins pose dietary health risks to humans and animals. These toxins inhibit peptidyl transferase activity which in turn limits eukaryotic protein synthesis. To assess the ability of intracellular antibodies (intrabodies) to modulate mycotoxin-specific cytotoxicity, a gene encoding a camelid single-domain antibody fragment (VH) with specificity and affinity for 15-AcDON was expressed in the methylotrophic yeast *Pichia pastoris*. Cytotoxicity and VH immunomodulation were assessed by continuous measurement of cellular growth. At equivalent doses, 15-AcDON was significantly more toxic to wild-type *P. pastoris* than was DON. In turn, DON was orders of magnitude more toxic than 3-AcDON. Intracellular expression of a mycotoxin-specific VH within *P. pastoris* conveyed significant (p = 0.01) resistance to 15-AcDON cytotoxicity at doses ranging from 20 to 100 µg·mL⁻¹. We also documented a biochemical transformation of DON to 15-AcDON to account for the attenuation of DON cytotoxicity at 100 and 200 µg·mL⁻¹. The proof of concept established within this eukaryotic system suggests that *in planta* VH expression may lead to enhanced tolerance to mycotoxins and thereby limit *Fusarium* infection of commercial agricultural crops.
5.2 Introduction

*Fusarium* head blight (FHB) of cereals and *Fusarium* Ear Rot of maize are caused by morphologically similar species (*F. graminearum*, *F. culmorum*, etc.) common throughout global agricultural regions. With few exceptions, *Fusarium* epidemics are characterized by cyclical and highly aggressive infection of commercial crops with economic impacts on food and feed industries that are immediate and far reaching. For example, losses associated with the most recent *Fusarium* outbreak in North America in the 1990s were estimated to range from $1.3 to 3.0 billion USD (Demcey Johnson et al., 2003).

A toxin class commonly found within agricultural commodities infected by *Fusarium* are trichothecene mycotoxins. Trichothecenes represent a highly diverse group of over 180 sesquiterpenoid low molecular weight (typically 200-500 Da) mycotoxins characterized by a tricyclic ring structure containing a double bond at C-9,10 and an epoxide group at C-13 (Desjardins, 2006). Regardless of size and structural composition, trichothecenes are potent inhibitors of eukaryotic protein synthesis with specific activity on ribosomal protein L3 within the 60S subunit resulting in inhibition of peptidyl transferase activity (Carter and Cannon, 1977; Rocha et al., 2005). Although the capacity to inhibit protein synthesis is regarded as central to trichothecene cytotoxicity (Rotter et al., 1996; Ueno, 1984), adverse effects on eukaryotic cells may actually be attributed to dysregulation of cellular signaling and alterations in downstream gene expression (Pestka, 2008). As a result, trichothecenes such as deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15-AcDON), and 3-acetyl-deoxynivalenol (3-AcDON) (Figure [Figure]...
are considered to be inherently hazardous food and feed borne contaminants (Desjardins, 2006; D’Mello et al., 1999).
transformation to less toxic forms: e.g., DON-glucosyl conjugate (Poppenberger et al., 2003) or to 3-AcDON (Alexander, 2008), and/or reduce intracellular concentrations to effectively limit mycotoxin exposure to sensitive cellular targets. Collectively, such research can be applied to impart novel mechanisms of trichothecene resistance in higher order plants.

Yeast is well suited as a eukaryotic model organism to identify and validate mechanisms involved in host plant resistance to mycotoxins (Mitterbauer and Adam, 2002; Mitterbauer et al., 2004; Poppenberger et al., 2003). Test systems based on yeast offer cost-effective convenience and flexibility as one can validate a wide range of novel detoxification mechanisms within a short period of time at a minimal cost using common / non-specialized lab equipment. Assessment of mycotoxin resistance mechanisms is likewise straightforward as reproducible treatment-specific effects can be precisely determined based on simple measurements of cellular growth and function over time.

Single-domain heavy chain antibody fragments (i.e., V\textsubscript{H}H) from the camelidae heavy chain IgG subfamily, are among the smallest functional recombinant antibody (rAb) fragments at 14-15 kDa. V\textsubscript{H}H fragments exhibit the same exquisite specificity as larger immunoglobulins, with added biochemical advantages of high solubility, stability, and robust expression in various recombinant systems (Holliger and Hudson, 2005; Muyldermans, 2001). V\textsubscript{H}H fragments have been generated against low molecular weight ligands (haptens) and toxins (e.g., Doyle et al., 2008). Based on favorable physico-chemical properties and efficacy against a wide range of antigens, single domain rAb fragments have been developed and tested as immuno-therapeutic reagents with
applications ranging from pharmacology (Holliger and Hudson, 2005; Saerens et al., 2008) to plant science (Jobling et al., 2003).

This paper demonstrates that intrabody expression of a $V_{H}H$ fragment isolated from a hyper-immunized phagemid library with affinity for 15-AcDON (Doyle et al., 2008) can impart real-time immunomodulation of mycotoxin-specific cytotoxicity within a model eukaryotic system. *Pichia pastoris* was selected as the host organism based on an expected high level expression of functional $V_{H}H$ intrabody fragments and anticipated sensitivity to 15-AcDON. This system was established as a ‘proof of concept’ to demonstrate that intrabody expression of recombinant $V_{H}H$ fragments could impart a novel means of mycotoxin-specific resistance.

### 5.3 Experimental Procedures

#### 5.3.1 $V_{H}H$ Genes

NAT-267 $V_{H}H$ DNA sequence (GenBank: EU676170.1) (Doyle et al., 2008) was used to design a *P. pastoris* codon optimized version of the gene to which was added 3’ HA and 6His epitope tags, as well as *Eco*RI and *Xba*I cloning sites at the respective 5’ and 3’ ends (GeneArt™, Toronto, ON, Canada). A non-specific $V_{H}H$ gene (B-24) isolated from a hyper-immunized phagemid llama library with confirmed non-specificity for 15-AcDON, or any other trichothecene mycotoxin, was used as a $V_{H}H$ intrabody control. Both $V_{H}H$ fragments were of the same immunoglobulin family and had similar molecular weights and isoelectric points. Like NAT-267, B-24 $V_{H}H$ DNA was PCR amplified to include 3’ HA- and 6His- epitope tags and restriction cloning sites *Eco*RI and *Xba*I with the following gene-specific primers: VHH-B24For (5’-GGAATTCCATG
CAGGTAAAGCTGGAGGAG (5’-GGTCTAGACCCTATGCT CGGCCGAACCGTAG-3’). A homology chart of nucleotide and peptide sequences of NAT-267 and B-24 \( V_{H} \)s is provided to show respective framework (FR) and complementarity determining regions (CDR), endonuclease sites and epitope tags (Fig. S1 in Supplementary Information). \( V_{H} \) DNA inserts (~ 0.46 kb) were ligated into pPICZB vector (Invitrogen Inc., Carlsbad, CA, USA). Constructs were sequenced by StemCore Labs (Ottawa, ON, Canada) using pPICZB-specific primers: 5’ AOX1For (5’-GACTGGTTCCAATTGACAAGC-3’) and 3’ AOX1Rev (5’-GCAAATGGCATTCTGACCATCC-3’).

5.3.2 Transformation

Expression constructs (Figure 5.2) were linearized with SacI and electroporated into \( P. \) *pastoris* strain KM71H (Invitrogen Inc., Carlsbad, CA, USA). Transformants were plated onto yeast extract, peptone, dextrose, sorbitol (YPDS) agar containing 100 \( \mu \)g·mL\(^{-1}\) zeocin and incubated for 3 days at 30°C until colonies formed. Ten colonies
from each transformant were re-streaked on yeast extract, peptone and dextrose (YPD) agar plates with 100 µg·mL⁻¹ zeocin to ensure pure clonal isolates.

5.3.3 Induction of V₁H Expression

Single colonies of V₁H and pPICZB control transformants were used to inoculate 5 mL YPD media. Cultures were grown overnight (30°C, 300 rpm). One mL of each culture was used to inoculate 100 mL of minimal glycerol medium with histidine (MGYH) and cultured in 1-L baffled flasks (30°C, 250-300 rpm) for 1 day. Cells were harvested with centrifugation (3,000g x 5 min.) at room temp. To induce V₁H expression, *Pichia* cells were resuspended in 20 mL of minimal methanol medium with histidine (MMH) and transferred to 125-mL baffled flasks and incubated at 30°C with 300 rpm shaking. Methanol (100%) was added to final conc. of 0.5% (% v/v) every 24 hours. Western blot analysis of ten V₁H (NAT-267 and B-24) transformants from 0, 24, 48, 72, 96, and 120 hours post-induction was used to select clones and induction time points corresponding to the highest overall protein expression.

5.3.4 Preparation of Soluble V₁H Extracts and Western Blot Analysis

Cell pellets were thawed on ice and resuspended in 100 µL lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 0.5% Triton-X 100, 1 mM DTT, 1X Roche’s Complete Protease Inhibitor Cocktail). Equal volumes of acid-washed 0.5-mm glass beads were added to each resuspended pellet, followed by successive 30-second cycles of vortexing and incubation on ice for a total of ten cycles. Cell lysate samples were clarified by centrifugation (14,000 rpm x 10 min.) at 4°C. One-hundred microliters of 2X-SDS loading buffer (BioRad Laboratories, Mississauga, ON, Canada) was added to the
supernatant followed by boiling (95°C x 5 min.) and electrophoresis on 12% SDS-PAGE gels. Samples were transferred to PVDF membrane (BioRad, Mississauga, ON Canada) and blocked overnight in 1% blocking reagent (Roche, Laval, QC, Canada) in TBS at 4°C. V_H intrabody fragments were detected by probing for 1 h with rabbit anti-HA IgG primary antibody (Sigma, St. Louis, MO) diluted 1:1000 in 0.5% blocking buffer followed by 2 washes with TBS + 0.1% Tween (TBST) and 2 washes with TBS + 0.5% blocking reagent. Secondary antibody (mouse anti-rabbit IgG horseradish peroxidase conjugate; Jackson, Immuno-Researcher Labs, West Grove, PA, USA), diluted 1:50,000 in 0.5% blocking buffer, was used to probe the membrane for 1 h, followed by 4 consecutive 15 min washes with TBST. V_H proteins were visualized with ECL Plus Western Blotting Detection System (GE Healthcare, Baie d’Urfé, QC, Canada). Transformants with the highest V_H intrabody expression levels were used in subsequent in vivo cytotoxicity assays.

5.3.5 Cytotoxicity Assays

Cells were induced as previously described. Forty-eight hours post induction, P. pastoris cells were diluted to an OD_{600} of 0.25 in YPD and incubated for 4 hours (30°C, 300 rpm). After this “recovery period”, cells were diluted to an OD_{600} of 0.1 in YPD, and immediately transferred (250 μL·well⁻¹) to an F96 microwell assay plate (Nalge Nunc Inc., Naperville, IL, USA). Pre-calibrated concentrations of the four ribotoxin treatments (15-AcDON, DON, 3-AcDON, or cycloheximide) prepared in DMSO and a DMSO only control were added to P. pastoris cells contained in the wells of assay plates. All treatments, respective controls, and cell-free wells containing only YPD media (i.e. blank
wells) were established in triplicate on each plate. Plates were sealed with Progene pressure-sensitive optical sealing film (Ultident, St.-Laurent, QC, Canada) prior to initiation of cytotoxicity assays. Cellular growth (30°C, 300 rpm) measured in real time based on measurement of OD$_{620}$ values at 25 min intervals, following 5 minutes of shaking (600 rpm) in a Polarstar Optima Microplate Reader (BMG Labtech, GmbH, Offenberg, Germany). Assay results were considered valid if similar and reproducible effects were observed in three separate experiments, conducted in triplicate under the same test parameters.

5.3.6 Data Analysis

Results were presented as *P. pastoris* growth curves over a 24-h time period. Mean (n = 3) OD$_{620}$ values for V$_{H}$H and pPICZB (empty vector) transformants used in ribotoxin and control treatments, minus OD$_{620}$ values of YPD media (blank) wells, were plotted against time (i.e. 25-min intervals). Standard error values and paired t-tests (p = 0.05 and 0.01) were used to assess statistically significant differences at the end of each 25-min time interval.

Time to doubling of initial *P. pastoris* OD$_{620}$ cellular growth values were calculated from cytotoxicity assay data to assess relative toxicity of each ribotoxin treatment. A comparative ranking of various ribotoxin treatments on *P. pastoris* growth was calculated based on time required to double OD$_{620}$ values of t = 0.0h pPICZB empty vector transformants.

Differential area under curve (ΔAUC) values were calculated to quantify relative differences in *P. pastoris* growth over the full time course of each cytotoxicity assay.
Relative differences in cellular growth were established by subtracting mean OD$_{620}$ values of pPICZB (empty vector) wells from corresponding values for V$_{H}$-transformants. ΔAUC values for each toxophore treatment were calculated based on addition of differences in cellular growth between V$_{H}$ and control transformants at each 25 min time interval across the full time course of each assay.

5.3.7 Quantitative Western Blot Analysis

20-mL cultures were centrifuged (1500g, 5 min, 4°C) 48 hours after methanol induction. Culture pellets were lysed and prepared for SDS-PAGE as described above. V$_{H}$ lysate samples (500 µL) were diluted 1:5; 1:25; and 1:50 in non-reducing SDS sample buffer (BioRad, Mississauga, ON, Canada). pPICZB vector only (control) samples were not diluted. A reference V$_{H}$ rAb fragment of a precisely-defined concentration, and of similar size to NAT-267 and B-24 V$_{H}$ with C-terminal HA and 6-His epitope tags, was used to establish a standard dilution series at final protein concentrations of: 300.0; 150; 75; 37.5; and 18.75 ng·lane$^{-1}$. To decrease the bias of Western blot band intensities, V$_{H}$ standard dilution series was prepared in P. pastoris cell lysate from pPICZB empty-vector sample.

Samples were loaded onto 12.5% SDS-PAGE gels and electrophoresed followed by transfer to a PVDF membrane (Millipore, Billerica, MA, USA) followed by a 1-h blocking with 3% non-fat skimmed milk in PBST. Mouse anti-6His IgG primary antibody (GE Healthcare, Piscataway, NJ, USA) diluted 1:2,500 in 3% milk was added to membranes for 1 h followed by 1 h of blocking and 3 consecutive washes with PBST. Alkaline phosphatase labeled goat-anti-mouse IgG conjugate (Cedarlane Laboratories,
Burlington, ON, Canada), diluted 1:3000 in blocking buffer, was used to probe the membrane for 1 h followed by 3 consecutive washes with PBST. \( V_{H} \) proteins were visualized using an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA, USA).

Quantitative Western blots were dried and photographed with an AlphaImager 3400 (Alpha Innotech Corporation, San Leandro, CA). Analysis was performed using the AlphaEaseFc software package (Version 7.0.1, Alpha Innotech Corp.). Densometric (Denso) values for a standard five data-point \( V_{H} \) dilution series (18.75 - 300 ng \( V_{H} \) · lane\(^{-1}\)) were used to produce regression equations. Denso values measured for 1:5; 1:25; and 1:50 dilutions of NAT-267 or B-24 \( V_{H} \) samples were expressed as: \( V_{H} \) mass per \( \mu L \) of \( P. \) pastoris cell pellet (ng·\( \mu L^{-1} \)), based on: \( V_{H} \) quantity (ng) (from standard regression equation) divided by cell pellet lysis volume loaded into each well (\( \mu L \)).

Cellular densities for each cytotoxicity assay were determined by multiplication of OD\(_{600}\) of 1.0 mL culture by 5 \( \times \) 10\(^7\) cells per mL culture (constant). \( V_{H} \) concentration (fg·cell\(^{-1}\)) was calculated as \( V_{H} \) mass per \( \mu L \) cell pellet (ng·\( \mu L^{-1} \)) (above) divided by cell density (number of cells·\( \mu L^{-1} \) culture). \( V_{H} \) intrabody concentration expressed as attoMole \( V_{H} \) per cell (aMol·cell\(^{-1}\)) was calculated based on: \( V_{H} \) mass per cell (fg·cell\(^{-1}\)) divided by molecular weight of NAT-267 or B-24 \( V_{H} \) proteins (16637 and 16976 Da, respectively). Finally, intracellular \( V_{H} \) molarity (\( \mu M \)oles \( V_{H} \) ·L\(^{-1}\)) was calculated as \( V_{H} \) concentration (aMol·cell\(^{-1}\)) divided by cell volume. \( P. \) pastoris cell volume was estimated to be 29 femtoliters per cell (fL·cell\(^{-1}\)) based on average of independent measurements of generic yeast-cell volume: 42, 37, 70, and 83 fL (Jorgensen et al., 2002;
Tyson et al., 1979; Sherman, 2000; and Tamaki et al., 2005; respectively) multiplied by 0.5 to account for an estimate that the cytosol represents 50% of total yeast cell volume (Biswas et al., 2003).

Mean and standard error values of intracellular $V_{HH}$ expression values were calculated based on five independent quantitative Western blot assays. Additional details are summarized in supplemental information (Table S5).

5.3.8 $V_{HH}$ Immunolocalization

 Cultures of $P$. $pastoris$ cells expressing either NAT-267 $V_{HH}$ or pPICZB (empty vector) were induced with methanol (as described). Cells (20 OD$_{600}$ units) were pelleted and washed three times with PBS and resuspended in 500 µL PBS. Fifty-µL aliquots of $P$. $pastoris$ cells were incubated in 500 µL fixative solution (1:1 acetone:methanol) for 20 minutes at -20°C. Cells were pelleted (1000g x 1 min), washed three times with PBS and resuspended in 500 µL PBS supplemented with Lyticase (5 U·µL$^{-1}$). After incubation (30°C, 15 min.), cells were pelleted (as described) and incubated in 500 µL permeabilization solution (3% BSA (bovine serum albumin), 0.5% Triton X-100 in PBS) for 30 minutes at room temp. After washing with incubation solution (1% BSA, 0.5% Tween 20), cells were probed with a 1:100 dilution of monoclonal anti-HA-FITC antibody (Sigma-Aldrich, Oakville, ON, Canada) for 1-2 h at room temp. Cells were mounted in PBS supplemented with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) stain (Sigma-Aldrich, Oakville, ON, Canada) at a final concentration of 1.3 µg·ml$^{-1}$. Cells were observed after 5 minutes using a Carl Zeiss LSM 510 Meta confocal microscope equipped with Diode 405 nm and Argon/2 line 488 nm lasers for excitation.
Emission images were taken 420-480 nm and 505-565 nm for DAPI and FITC, respectively.

5.3.9 Mycotoxin Biotransformation Assays

After 48 hours of induction (see above), cultures were diluted to an OD$_{600}$ of 0.25 in YPD and incubated for 4 h (30°C, 300 rpm). Cultures were diluted to OD$_{600}$ 0.1 in YPD, and 2.5-mL aliquots of each culture were transferred to 50-mL tubes. Highly purified DON was added to respective samples to a final concentration of 100 µg·mL$^{-1}$ followed by incubation (30°C, 300 rpm). Culture samples (0.5 mL) were taken at intervals of 30 min., 16 and 24 h after mycotoxin addition and used to measure OD$_{600}$ and culture pH. Samples were pelleted (3500g, 5 minutes). Supernatant was removed and transferred to 2.0-mL tubes. Samples were frozen in liquid nitrogen and stored at -80°C. Lysate samples were prepared from cell pellets (as described above). All samples were split to enable concurrent anti-HA Western blot analysis (as described above) and mycotoxin biotransformation assays.

Supernatant, cell lysate, and pellet of selected samples were washed with dd H$_2$O and passed through 0.8 µm filter (Millipore). Filtrates were passed through Chromosep C$_{18}$ columns (C$_{18}$ Sep-Park Cartridge, Waters Corp., Millford, MA), pre-washed with 10 mL of 100 % (w/v) HPLC grade methanol and 10 mL of dd H$_2$O. After washing with dd water (10 mL), mycotoxin fractions were eluted with 10 ml of 100 % (v/v) HPLC grade methanol and dried under a stream of N$_2$. Selected culture samples (as described in Table 5.82) were diluted in 0.5 mL HPLC grade methanol (100%) and 50.0-µL subsamples were used for HPLC analysis.
Trichothecenes were separated on a Shimadzu HPLC system equipped with a 150 x 4.6 mm C$_{18}$ column (5 µm packing size) and a Hichrome UV flow cell. Standards and samples were eluted at a flow rate of 1.0 mL·min$^{-1}$ with a 20 min linear gradient of 95% solvent A (degassed dd H$_2$O): 5% solvent B (acetonitrile) to 40% solvent A: 60% solvent B. The column was washed from 20-25 min by ramping the gradient to 100% solvent B, holding at 100% B for 5 min and then returning to the starting conditions over 2 min. Assessment of DON bio-transformation was based on assessment of retention times of sample peaks relative to trichothecene standard peaks.

GC/MS analysis, based on a standard Canadian Food Inspection Agency approved method (Schwadorf and Müller, 1991), was used to further characterize and validate HPLC samples for structural composition of trichothecene analytes. Biotransformation samples from HPLC analysis were derivatized with 1.5 mg·mL$^{-1}$ dimethylaminopyridine (DMAP) in toluene:acetonitrile (95:5) solution and trifluoroacetic anhydride (TFAA). This solution was heated at 60°C for 30 minutes, and neutralized twice with 5% KH$_2$PO$_4$. Derivatized samples were analyzed on a Saturn 2000 GC/Ion-trap mass spectrometer equipped for chemical ionization using acetonitrile.

$^1$H NMR spectra of pooled $P$. pastoris lysate samples and subsequent HPLC fractions of those samples were obtained on a Bruker AM 500 NMR spectrometer in CDCl$_3$. Chemical shifts are referenced to residual CHCl$_3$ at 7.24 ppm for $^1$H spectra and reported (δ) relative to TMS.
5.4 Results

5.4.1 Sensitivity to Ribotoxin Treatments

The structure, composition, and molecular weight of the mycotoxins used in these experiments are shown in Figure 5.1. The sensitivity of wild-type P. pastoris to trichothecone and cycloheximide (control) ribotoxin treatments, was governed by dose and chemical structure tested. Treatments based on 15-AcDON resulted in the most immediate and largest overall reduction of cellular growth (Table 5.1).

Table 5.1 Sensitivity of P. pastoris (KM71H) pPICZB transformants to various ribotoxin treatments tested. Standard deviations (σ) for each mean value shown in brackets.

<table>
<thead>
<tr>
<th>Dose (µg∙mL⁻¹)</th>
<th>15-AcDON</th>
<th>DON</th>
<th>3-AcDON</th>
<th>Cycloheximide</th>
<th>L.S.D. ††</th>
</tr>
</thead>
<tbody>
<tr>
<td>0†</td>
<td>205 (2.2)</td>
<td>221 (14.4)</td>
<td>219 (21.7)</td>
<td>221 (14.4)</td>
<td>14.5</td>
</tr>
<tr>
<td>20</td>
<td>322 (4.4)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>384 (4.4)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>439 (6.6)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>494 (8.8)</td>
<td>308 (7.2)</td>
<td>190 (124)</td>
<td>283 (7.2)</td>
<td>71.7</td>
</tr>
<tr>
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<td>417 (47.3)</td>
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</tbody>
</table>

n.d. not determined
† DMSO, ribotoxin-free media.
†† Least Significant Difference (p=0.05)

P. pastoris was comparatively less sensitive to equivalent doses of DON and cycloheximide. It was not possible to establish a dose-specific sensitivity to 3-AcDON
relative to toxin-free DMSO media as cellular growth was not inhibited at the highest concentration (200 µg·mL⁻¹) (Table 5.1).

5.4.2 Transformation and V₃H Intrabody Expression

*P. pastoris* KM71H cells were successfully transformed with linearized NAT-267 (treatment) or B-24 (control) V₃H DNA ligated into expression vector pPICZB (Figure 5.2). Lysate fractions of methanol-induced transformants were assessed by anti-HA epitope Western blot analysis and robust intracellular V₃H expression was confirmed by the presence of a 17 kDa band at 48 and 72 hours post methanol-induction (data not shown).

5.4.3 Quantitative Western Blot Assay

Mean levels of soluble intracellular V₃H expression (t = 0h) transformants tested within cytotoxicity assays were determined by quantitative western blot analysis (Figure 5.3). The initial concentration of NAT-267 V₃H in *P. pastoris* cells within cytotoxicity assays of 15-AcDON (Figure 5.4) was 4.01 ± 0.31 aMol V₃H·cell⁻¹ (Table 5.2).

![Figure 5.3](image_url) Quantitative Western blot analysis of V₃H intrabody expression within cytotoxicity assays. Blot 1 is V₃H standard dilution series from 300.0 to 18.75 ng·well⁻¹. Blot 2 is NAT-267 V₃H expression for cytotoxicity assay #1 (Figure 5.4). Blot 3 is NAT-267 V₃H expression for cytotoxicity assay #2 (Figure 5.5). Blot 4 is B-24 (control) V₃H expression for cytotoxicity assay #3 (Figure 5.6). BioRad Molecular weight marker (MW) indicated on the left. Protein dilutions are shown along bottom of blots 2 to 4.
Accounting for an estimated mean *P. pastoris* cytosol volume of 29 fL·cell⁻¹ (as previously described), initial expression of NAT-267 VᵢH was equivalent to an intracellular concentration of 138 ± 10.8 µMoles VᵢH·L⁻¹. Likewise, mean NAT-267 intrabody concentration within cells used in cytotoxicity assays of DON, 3-AcDON, and cycloheximide (Figure 5.5) was 3.18 ± 0.42 aMol·cell⁻¹ or 110 ± 14.4 µMol·L⁻¹ (Table 5.2). The average initial concentration of B-24 control VᵢH intrabody in cytotoxicity assays of 15-AcDON and cycloheximide (Figure 5.6) was 1.93 ± 0.14 aMol·cell⁻¹ or 66.6 ± 4.3 µMol·L⁻¹ (Table 5.2).

**Table 5.2** Mean VᵢH intrabody concentration (t = 0 h) within *P. pastoris* transformants used in cytotoxicity assays (Figures: 5.4, 5.5, 5.6). Values derived from data generated from quantitative Western blot assays (Figure 5.3) as summarized in Table 5.S1. VᵢH concentration values expressed in femtogram and attoMol VᵢH per cell, as well as µMol·L⁻¹ VᵢH equivalent within *P. pastoris* cytosol. Standard Error for all means (n = 9) is shown in parenthesis.

<table>
<thead>
<tr>
<th>VᵢH Gene</th>
<th>Cytotoxicity Assay</th>
<th>VᵢH Intrabody Concentration</th>
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<tbody>
<tr>
<td></td>
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<td>fg·cell⁻¹</td>
</tr>
<tr>
<td>NAT-267</td>
<td>15-AcDON (Figure 5.4)</td>
<td>66.8 (± 5.2)</td>
</tr>
<tr>
<td>NAT-267</td>
<td>DON, 3-AcDON Cycloheximide (Figure 5.5)</td>
<td>53.0 (± 7.0)</td>
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<tr>
<td>B-24</td>
<td>15-AcDON Cycloheximide (Figure 5.6)</td>
<td>32.8 (± 2.3)</td>
</tr>
</tbody>
</table>
5.4.4 Cytotoxicity Assays

Intrabody V_{H}H expression did not influence the cellular growth of transformed *P. pastoris* cultures in mycotoxin-free DMSO media, as OD\textsubscript{620} values and overall growth rate of NAT-267 and B-24 V_{H}H transformants were similar to pPICZB empty-vector control cells (Figures: 5.4, 5.5, 5.6).

5.4.4.1 15-AcDON

Significant differences (p = 0.01) in cellular growth were observed between NAT-267 V_{H}H and pPICZB control transformants grown in media spiked with 20-100 \mu g\cdot mL\textsuperscript{-1} 15-AcDON (Figure 5.4). The impact of NAT-267 V_{H}H intrabody expression on cellular growth was dependent on the concentration of 15-AcDON used. At the lowest dose (20 \mu g\cdot mL\textsuperscript{-1}), growth of NAT-267 V_{H}H expressing cells was similar to cell lines grown in DMSO (control) media.

Time to significantly improved (p=0.01) growth of NAT-267 V_{H}H intrabody transformants was dose-dependent. Significant (p=0.01) differences in OD\textsubscript{620} values were observed after 7.5 hours of growth for cells cultured in medium supplemented with 15-AcDON at both 20 and 50 \mu g\cdot mL\textsuperscript{-1} (Figure 5.4). Assays spiked with 30, 40, and 100 \mu g\cdot mL\textsuperscript{-1} 15-AcDON had significant differences in OD\textsubscript{620} values after 4.6, 2.9, and 11.3 hours of growth, respectively. No differences in OD\textsubscript{620} values were observed between NAT-267 V_{H}H and control transformants at 200 \mu g\cdot mL\textsuperscript{-1} 15-AcDON (Figure 5.4).

Differential area under curve (ΔAUC) analysis was used to quantify relative differences in cellular growth between NAT-267 and B-24 (control) V_{H}H transformants.
Figure 5.4 Cytotoxicity assays showing the effect of various concentrations (20 to 200 µg·mL$^{-1}$) of 15-AcDON on the growth of P. pastoris pPICZB and NAT-267 V$_{H}$H transformants as measured by optical density at 620 nm (OD$_{620}$). Differential Area Under Curve (ΔAUC) values for NAT-267 V$_{H}$H minus pPICZB empty vector are shown on each graph. At all concentration of 15-AcDON (except 200 µg·mL$^{-1}$) where blue versus red symbols are used, there was a significant difference ($p=0.01$) at each time between NAT-267 and pPICZB transformants.
The largest ΔAUC value (5.25) corresponded to NAT-267 V_{H}H expressing *P. pastoris* grown with 15-AcDON at 30 µg·mL⁻¹. Cytotoxicity assays based on 15-AcDON at 20, 40, and 50 µg·mL⁻¹ had similar ΔAUC values (4.48; 4.50; and 4.45, respectively) ([Figure 5.4](#)). Accordingly, assays at 100 µg·mL⁻¹ produced a substantially lower ΔAUC value (1.20) while NAT-267 V_{H}H intrabody expression conferred no immunomodulation when spiked with 200 µg·mL⁻¹ 15-AcDON ([Figure 5.4](#)).

### 5.4.4.2 DON, 3-AcDON, Cycloheximide

NAT-267 V_{H}H intrabody expression resulted in a dose-dependent response to DON. Although no beneficial impact on cellular growth was observed in cultures supplemented with 50 µg·mL⁻¹ DON, NAT-267 V_{H}H resulted in significantly-improved cellular growth at 100 and 200 µg·mL⁻¹ DON relative to pPICZB control transformants. Comparative differences in growth were greater for yeast spiked with 100 relative to 200 µg·mL⁻¹ DON (ΔAUC values = 2.11 and 1.68, respectively). In addition, time to significant differences in OD₆₂₀ values was several hours later for the higher dose ([Figure 5.5A](#)).

Differences in cellular growth between NAT-267 V_{H}H and pPICZB control *P. pastoris* cells could not be established for cytotoxicity assays spiked with 3-AcDON because this trichothecene had little or no adverse effect on *P. pastoris* growth ([Table 5.1](#)). Assays based on cycloheximide (control ribotoxin) exhibited a dose-dependent effect on cellular growth; however, no differences were observed between *P. pastoris* NAT-267 V_{H}H and pPICZB transformants spiked with doses of 50, 100, and 200 µg·mL⁻¹ ([Figure 5.5B](#)).
**Figure 5.5** Cytotoxicity assays showing the effect of various concentrations (50 to 200 µg·mL⁻¹) of DON (A) and cycloheximide (B) on the growth of *P. pastoris* pPICZB and NAT-267 V₁₁H transformants as measured by optical density at 620 nm (OD₆₂₀). Differential Area Under Curve (ΔAUC) values for NAT-267 V₁₁H minus pPICZB empty vector are shown for each concentration of DON. ΔAUC values for cycloheximide treatments were nil. At all concentrations of DON where green versus red symbols are used, there was a significant difference (p=0.01) at each time between the NAT-267 and pPICZB transformants; this was not the case for all cycloheximide treatments.
5.4.4.3 B-24 V_{H}H Control

Expression of B-24 control V_{H}H intrabody had no effect on cellular growth rate relative to pPICZB control transformants in DMSO or in media supplemented with either 15-AcDON or Cycloheximide (Figure 5.6). These results suggest that trichothecene-specific immunomodulation imparted by NAT-267 V_{H}H intrabody expression was trichothecene-specific, as Pichia cells expressing a non-specific V_{H}H intrabody were equally as sensitive as pPICZB control transformants.

5.4.5 V_{H}H Immunolocalization

NAT-267 V_{H}H intrabody expression and distribution within P. pastoris transformants was further validated with fluorescent confocal microscopy (Figure 5.7). Detection of the HA-tag on NAT-267 V_{H}H intrabody fragments within transformants grown in YPD media to OD_{600} = 20 further confirmed robust intrabody expression within our test system.

5.4.6 Mycotoxin Biotransformation Assays

Given that NAT-267 V_{H}H was confirmed previously to have binding affinity limited to 15-AcDON (Doyle et al., 2008), we were surprised to observe a dose-dependent amelioration to DON cytotoxicity within NAT-267 V_{H}H transformants (Figure 5.5A). To validate this observation and re-confirm in vivo antigen specificity of NAT-267 V_{H}H intrabody expression, we assessed the potential biotransformation of DON within our P. pastoris test system by spiking with DON. HPLC analysis of 16-h and 24-h cultures grown in DON-supplemented YPD media consistently yielded another peak in addition to the DON peak at ~ 5.85 min (Table 5.S2). This peak appeared at ~
Figure 5.6 Cytotoxicity assays showing the effect of various concentrations (50 to 200 µg·mL\(^{-1}\)) of 15-AcDON (A) and cycloheximide (B) on the growth of \textit{P. pastoris} pPICZB and non-specific B-24 V\(_{12}\)H transformants as measured by optical density at 620 nm (OD\(_{620}\)). Differential Area Under Curve (ΔAUC) values for B-24 V\(_{12}\)H minus pPICZB empty vector for both 15-AcDON and cycloheximide are nil. At all concentrations of DON and cycloheximide there was no significant difference (p=0.01) at each time between the B-24 and pPICZB transformants.
Figure 5.7 Representative confocal microscopy photographs of *P. pastoris* (KM71H) cells isolated, washed and immuno-probed with anti-HA epitope mAb-FITC conjugate and DAPI stain after growing in YPD media to OD$_{600}$ = 20. **A** = Control, pPICZB empty vector (control). **B** = *P. pastoris* transformants expressing NAT-267 V$_{1H}$H intrabody shown by fluorescent green cells.

9.5 min and matched the retention time of 15-AcDON (Table 5.S2). HPLC chromatograms of cell lysate and supernatant samples of pPICZB and NAT-267 V$_{1H}$H transformants provided clear evidence of a metabolic conversion of DON to 15-AcDON. No trichothecenes were found within cell pellet fractions (Table 5.S2). HPLC analysis revealed no evidence of DON-glucosyl metabolites or any structurally-similar compounds within samples tested. GC/MS analysis confirmed the presence of 15-AcDON within pooled cell lysate samples (data not shown). The GC retention time and mass spectrum of the peak confirmed the presence of 15-AcDON and absence of 3-AcDON within samples tested (data not shown). Finally, the $^1$H NMR spectra of the total filtrate extract
was dominated by DON and other impurities from the medium. However, the presence of 15-AcDON was confirmed in the NMR spectrum of the HPLC fraction isolated at ~9.5 min from the pooled lysate samples (Figure 5.8).

**Figure 5.8** Representative 500 mHz $^1$H NMR spectrum of A = 15-AcDON Standard. B = HPLC fraction of *P. pastoris* cell lysate sample (24 hrs). Unique chemical shifts of 15-AcDON are labeled due to H-11 (4.87 ppm), H-7 (4.81 ppm), and H-15 (4.21 ppm) protons which confirm the presence of 15-AcDON within cell lysate.
5.5 Discussion

Various strategies have been used to develop plants with enhanced resistance to trichothecene cytotoxicity and associated *Fusarium* pathogenesis. These approaches tend to be based on mechanisms which alter host cellular targets (Harris and Gleddie, 2001; Mitterbauer et al., 2004), reduce mycotoxin cytotoxicity (Alexander, 2008; Poppenberger et al., 2003), or enhance innate host resistance through crop breeding techniques (Snijders, 2004). Our goal was to evaluate the ability of a mycotoxin-specific VhhH intrabody to immuno-modulate, or attenuate, the cytotoxic effects of 15-AcDON using a yeast model.

Aside from application as a bioassay-indicator organism (Abolmaali et al., 2008; Binder, 1999), several species of yeast have been used to evaluate the *in situ* function of various trichothecene-specific genes (Alexander et al., 1999; Poppenberger et al., 2003) or assess efficacy of mycotoxin-specific transgenes prior to *in planta* application (Mitterbauer and Adam, 2002; Mitterbauer et al., 2004; Poppenberger et al., 2003). The methylotrophic yeast *P. pastoris* (strain KM71H) was selected as our eukaryotic model based on expected sensitivity to target ribotoxins and known capacity to express high levels of functional heterologous proteins (Cereghino and Cregg, 2000). We demonstrated that it was possible to transform, assess, and validate *P. pastoris* cells within a matter of weeks, thus creating an ideal platform for screening mycotoxin-specific constructs before *in planta* evaluation.

Trichothecene cytotoxicity is determined by the C-12,13 epoxide group common to this class of mycotoxins (Carter and Cannon, 1977; Rocha et al., 2005; Rotter et al.,
However, the number and position of hydroxyl and acetyl-ester groups on the trichothecene structure can also influence the mechanism of protein synthesis inhibition and relative toxicities within eukaryotic cells (Betina, 1989; Desjardins et al., 2007; Ehrlich and Daigle, 1987). In this regard, we found that the position of an acetyl-ester group on either carbon 3 or 15 of DON (Figure 5.1) had a profound effect on their cytotoxicity to *P. pastoris* (Table 5.1). Our findings align with results established in other yeast species where 15-AcDON was significantly more toxic than DON (Mitterbauer et al., 2004; Poppenberger et al., 2003), which was more toxic than 3-AcDON (Binder et al., 1997). These results also support findings of structure-activity studies established in planta that demonstrated 15-AcDON is more toxic than DON (Desjardins et al., 2007; Poppenberger et al., 2003) and 3-AcDON (Alexander, 2008; Desjardins, 2006). Our observation of no measurable cytotoxicity of 3-AcDON is in agreement with previous studies which demonstrated that addition of an acetyl group at C-3 (Figure 5.1) serves to eliminate cytotoxicity as part of a metabolic defence mechanism during trichothecene synthesis (Kimura et al., 1998a; Kimura et al., 2006).

Our results also quantify relative differences in trichothecene cytotoxicity over time, not at a specific assay endpoint. Our *P. pastoris* model system enables real-time evaluation of cellular effects during the initial phases of ribotoxin exposure. We propose that this biological system is very sensitive (i.e. compared to whole plant systems) and shows clear effects on cellular growth within the first hours of exposure thereby indicating the potency of trichothecene-mediated cytotoxicity.
A sequential two-step process was required before commencement of each cytotoxicity assay. First, transformants were induced with methanol for 48h. After induction of V_{H}H intrabody expression, *P. pastoris* cells were transferred to YPD media for a 4-h ‘recovery period’ to re-stimulate cellular growth. Mycotoxin or cycloheximide (control) treatments were then added (at t = 0h) to initiate each cytotoxicity assay. Given the well-established negative impact on eukaryotic protein synthesis, it was an obvious necessity to induce V_{H}H expression prior to addition of ribotoxin treatments. The 4-h recovery in YPD media was required because induction conditions did not support an optimal cellular division and growth over time. We also noted that, by virtue of its design, our test system ensured a fixed ratio of V_{H}H antibody to ribotoxin hapten throughout the course of each cytotoxicity assay. We recognized the potential disadvantage of culturing induced cells in YPD as leading to a continuous dilution of V_{H}H concentration within the cytosol of rapidly dividing cells. However, we sought to maintain a fixed ratio of Ab:Ag within each microtiter well to ensure unbiased measurements of overall cellular growth.

Soluble NAT-267 and B-24 control V_{H}H intrabody fragments were well expressed within *P. pastoris*. Average protein expression levels (Table 5.2) were found to be much higher than previously reported in *P. pastoris* (Gurkan et al., 2004; Omidfar et al., 2007; Rahbarizadeh et al., 2006). Furthermore, images generated by confocal immunomicroscopy confirmed that NAT-267 V_{H}H fragments were well distributed within the cytosol of *P. pastoris* transformants even after growth to a very high OD\textsubscript{600} (Figure 5.7).
Our principal hypothesis was that the expression of mycotoxin-specific \( V_{\text{H}} \) intrabody fragments would effectively sequester (i.e., neutralize) bio-available 15-AcDON concentrations within the cell and thereby limit cellular toxicity, thereby resulting in enhanced growth of \( P. \text{pastoris} \). \( V_{\text{H}} \)-mediated mycotoxin binding was assessed by time-to-significant immunomodulation and \( \Delta \text{AUC} \) values between \( V_{\text{H}} \) transformants and pPICZB (control) cell lines for each ribotoxin treatment tested (Figures 5.4 – 5.6). The initial doses of 15-AcDON tested are generally equivalent to those found during \textit{Fusarium} infection of agricultural crops. For example, intracellular levels of DON have been reported at concentrations ranging from 13.3 to 88.7 \( \mu \text{g} \cdot \text{g}^{-1} \) within \textit{F. graminearum} inoculated cereal tissue (Del Ponte et al., 2007; Mudge et al., 2006).

Attenuation of 15-AcDON activity clearly demonstrates that NAT-267 \( V_{\text{H}} \) intrabody expression conveys significant (\( p = 0.01 \)) resistance to mycotoxin-specific cytotoxicity (Figure 5.4) by regulating the availability of free and \( V_{\text{H}} \)-bound 15-AcDON. Consequently, results were dose-dependent as time to immunomodulation was optimal for 30 and 40 \( \mu \text{g} \cdot \text{mL}^{-1} \) 15-AcDON, when compared to the lower (20 \( \mu \text{g} \cdot \text{mL}^{-1} \)) and higher concentrations (i.e., 50 and 100 \( \mu \text{g} \cdot \text{mL}^{-1} \)) of the toxophore. Sequestration of 15-AcDON was substantially reduced and delayed at concentrations of 100 \( \mu \text{g} \cdot \text{mL}^{-1} \) while at 200 \( \mu \text{g} \cdot \text{mL}^{-1} \) there was no benefit associated with \( V_{\text{H}} \) expression (Figure 5.4).

NAT-267 \( V_{\text{H}} \) intrabody immuno-modulation was trichothecene-specific as no attenuation of 15-AcDON toxicity was observed in assays supplemented with control ribotoxin (cycloheximide) (Figure 5.5). It was not possible to assess the effect of NAT-267 \( V_{\text{H}} \) on the cytotoxicity of 3-AcDON because all doses tested were not toxic to \( P. \)
pastoris cells (Table 5.1). Further confirmation of NAT-267 V_{H}H specificity was shown with cytotoxicity assays using *P. pastoris* transformants expressing a control V_{H}H intrabody, i.e., B-24. Furthermore, there was no immunomodulation of either 15-AcDON- or cycloheximide-specific cytotoxicity when pPICZB control cells were used (Figure 5.6).

The attenuation of DON cytotoxicity at 100 and 200 µg·mL^{-1} (Figure 5.5) was an unexpected result since we previously-reported that NAT-267 V_{H}H has no affinity for DON (Doyle et al., 2008). *In vivo* sequestration of DON was explained by confirmation of bio-transformation of DON to 15-AcDON in *P. pastoris* cell lysate and culture supernatant samples of NAT-267 and pPICZB transformants (Table 5.S2; Figure 5.8). Data mining of the genome of NRRL Y-11430 *P. pastoris* (the parent strain of KM71H) by Integrated Genomics Inc. (Chicago, IL, USA) revealed no similarities or related homologs to a previously characterized fungal gene responsible for acetylation of DON to 15-AcDON (McCormick et al., 1996). We therefore attributed this biochemical conversion to a previously-uncharacterized acetyl-transferase gene within the *P. pastoris* genome.

To predict the impact of NAT-267 V_{H}H intrabody binding to reduce effective 15-AcDON toxin concentrations within the cytosol, we adopted a previously described (Almquist et al., 2004) mass-balance model based on the following equation.

\[
[\text{Bound Toxin}] = \frac{[V_{H}H] \times [\text{Total Toxin}]}{([V_{H}H] + K_D)}
\]
We assumed linear, dose-independent binding of V_H to 15-AcDON within an aqueous environment where 1 g = 1 mL. The dissociation constant ($K_D$) of NAT-267 V_H was taken as 1.24 µM (Doyle et al., 2008) with 1:1 stoichiometry of Ab:Ag binding. NAT-267 ([V_H]) concentration within the *P. pastoris* cytosol was estimated to be 138 µmol·L$^{-1}$ (Tables 5.2, 5.S1). We assumed optimal disulfide bond formation and post-translational V_H folding within the endoplasmic reticulum and cytosol (Ellgaard, 2004) with no NAT-267 V_H intrabody leakage from the *P. pastoris* cell membrane. The concentration of 15-AcDON within the cytosol was taken as equivalent to culture media, and we also assumed that there were no trichothecene targets outside of the cells.

Based on total 15-AcDON concentration of 50 µg·mL$^{-1}$ (or 148 µM) (Figure 5.4), this model predicts $(138 \mu M \times 148 \mu M) \div (138 \mu M + 1.24 \mu M) = 147 \mu M$ of 15-AcDON (or $>99.9\%$) toxin was bound and $\sim 1.3\mu M$ (or $<0.1\%$) of 15-AcDON was free. This simple model indicates that 15-AcDON cytotoxicity at 50 µg·mL$^{-1}$ should be completely eliminated by expression of NAT-267 V_H; however, although the effects of 15-AcDON were significantly ameliorated by V_H expression at this concentration, they were not completely eliminated (Figure 5.4).

A more complete assessment of NAT-267 V_H immunomodulation should also account for *in situ* competition effects and binding affinity between 15-AcDON and its major cellular target, i.e., ribosome binding. Binding affinity is also an important consideration because NAT-267 V_H has a relatively low affinity ($K_D = 1.24 \mu M$) for 15-AcDON that is mediated by weak, non-covalent interactions (e.g., van der Waals forces, hydrogen bonding, etc.) (Maynard and Georgiou, 2000). Due to the rapid dissociation rate
constant for NAT-267, we hypothesize that 15-AcDON is subject to continuous turnover in terms of mycotoxin binding to the V_{H}H. In other words, the efficiency of NAT-267 V_{H}H in terms of limiting the cytotoxicity of 15-AcDON is limited by the short “residence time” of hapten binding to the V_{H}H (Copeland et al., 2006). Further evidence for this hypothesis resides in the fact that even when the V_{H}H intrabody is expressed in excess (138 μmol·L^{-1}, Table 5.2) compared to 15-AcDON, i.e. at 89 μM (30 μg mL^{-1}), NAT-267 cannot fully immunomodulate the cytotoxic effects of 15-AcDON when compared to controls (Figure 5.4). Thus, at higher doses ≥30 μg·mL^{-1} of 15-AcDON, we postulate that the V_{H}H simply cannot compete with a stronger and quite possibly longer residence time of 15-AcDON on the 60S ribosomal protein subunit L3 (Rpl3) of *P. pastoris*.

We also assert that, at very high doses, 15-AcDON may cause other potentially irreversible effects such as membrane disruption, inhibited RNA and DNA synthesis, and various other apoptotic effects (reviewed in Rocha et al., 2005; Rotter et al., 1996; Ueno, 1984) which may also severely limit cellular growth. Thus, to accurately determine the efficacy of NAT-267 V_{H}H intrabody in *P. pastoris* cells one must account for the dynamic nature of mycotoxin-mediated cytotoxicity and *in vivo* binding kinetics of 15-AcDON for other cellular binding targets. Our assertion that attenuation of trichothecene-specific cytotoxicity is not a simple process is in agreement with previous work which demonstrated only partial remediation of DON cytotoxicity based on *in planta* expression of an altered Rpl3 target protein (Harris and Gleddie, 2001; Mitterbauer et al., 2004) or the expression of *Tri*101 (Alexander, 2008, Kimura et al., 1998a).
Most phytopathogenic *Fusarium* species are believed to produce 15-AcDON or 3-AcDON as acetylated precursors to DON (Mitterbauer and Adam, 2002). Production of each acetylated compound has been described as geography-dependent; 3-AcDON chemotypes dominate in Asia and Europe, while *Fusarium* species which produce 15-AcDON are more prevalent in North America (Goswami and Kistler, 2004; Moss and Thrane, 2004). Therefore, the accumulation of DON within plant tissue is believed to be a metabolic process conferred by fungal and plant carboxyl-esterases which continuously deacetylate either 15-AcDON or 3-AcDON within plant tissue (Mitterbauer and Adam, 2002). Confirmation of NAT-267 efficacy observed in this work is very significant since 15-AcDON was the most cytotoxic compound tested. If 15-AcDON is as toxic to plants as it is to yeast then expressing NAT-267 V_HH within the cytosol before trichothecene accumulation may help limit *in vivo* pathogenesis and metabolism to DON during *Fusarium* infection of plants such as corn and wheat.

Future experiments will focus on the development of an anti-15-AcDON V_HH with an improved dissociation constant ($K_D$) to ensure a stronger association between the V_HH and target ligand for improved *in vivo* efficacy (Copeland et al., 2006). It would also be of great interest to develop and test novel V_HH fragments with affinity for various other trichothecenes (e.g., neosolaniol, diacetoxyscripenol, T-2 toxin, etc.) and various other mycotoxin classes (e.g., fumonisins, aflatoxins, etc.) using this test system. A logical subsequent application would be constitutive *in planta* expression of optimized mycotoxin-specific V_HH fragments, possibly with catalytic activity, to bind and deactivate/degrade mycotoxins during critical initial periods of plant pathogenesis.
5.6 Acknowledgements

We would like to express our sincere appreciation to Dr. Barbara Blackwell at Agriculture and Agri-Food Canada Ottawa for assistance, insights, and expertise in HPLC, GC/MS, and NMR assays to characterize mycotoxin biotransformation samples. We are also indebted to colleagues at the Canadian Food Inspection Agency for GC/MS analysis of biotransformation samples. This research was supported by grants to JCH from the Natural Sciences and Engineering Research Council of Canada, the Canada Research Chairs Program, and the Ontario Ministry of Agriculture, Food, and Rural Affairs.
Figure 5.51 Homology chart of NAT-267 and B-24 V_{H} fragments. Framework regions (FR) are outlined in black while complementarity determining regions (CDR) 1, 2, and 3 are outlined in blue. Cysteine residues at C23 and C97 responsible for disulfide bond V_{H} folding are shown in red. XXX denotes extra codon within CDR3 of NAT-267 gene. Restriction endonuclease sites, epitope tags (6HIS = green, HA = pink) and stop codons (*) are marked accordingly. NAT-267: Theoretical pI for NAT-267 and B-24 V_{H} = 9.00 and 9.17, respectively (ExPASy).
Table 5.S1: Raw data and calculations of \( \text{V}_{\text{H}} \) intrabody expression in *Pichia pastoris* transformants derived from quantitative Western blot analysis (Figure 6.3) and as summarized in Table 6.2.

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<tr>
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<tr>
<td>1 / 5</td>
<td>192.3</td>
<td>96.15</td>
<td>34.13</td>
<td>31.75</td>
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<td>1 / 25</td>
<td>60.90</td>
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<td>54.05</td>
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<tr>
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<td>305.30</td>
<td>152.65</td>
<td>54.19</td>
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<td>114.11</td>
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<td>101.27</td>
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<td>1 / 50</td>
<td>53.84</td>
<td>269.19</td>
<td>95.56</td>
<td>88.90</td>
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<td>184.2</td>
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<tr>
<td>1 / 5</td>
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<td>48.39</td>
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<td>2.705</td>
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<td>88.57</td>
<td>221.43</td>
<td>78.61</td>
<td>73.12</td>
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<td>50.67</td>
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<td>89.93</td>
<td>83.66</td>
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<td></td>
<td>5.23</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Cytotoxicity Assay-1: NAT-267 vs. 15-AcDON (Figure 6.4)

| Blot 1          |                                 |                                 |                                 |                                 |                                 |                                 |
| 1 / 5           | 127.9                           | 63.94                           | 22.70                           | 19.48                           | 1.171                           | 40.4                            |
| 1 / 25          | 25.11                           | 62.78                           | 22.29                           | 19.13                           | 1.150                           | 39.6                            |
| Blot 2          |                                 |                                 |                                 |                                 |                                 |                                 |
| 1 / 5           | 238.9                           | 119.5                           | 42.42                           | 36.41                           | 2.188                           | 75.5                            |
| 1 / 25          | 107.5                           | 268.8                           | 95.41                           | 81.89                           | 4.922                           | 169.7                           |
| 1 / 50          | 69.19                           | 346.0                           | 122.8                           | 105.42                          | 6.336                           | 218.5                           |
| Blot 3          |                                 |                                 |                                 |                                 |                                 |                                 |
| 1 / 5           | 148.6                           | 74.3                           | 26.38                           | 22.64                           | 1.361                           | 46.9                            |
| 1 / 25          | 85.36                           | 213.4                           | 75.75                           | 65.03                           | 3.908                           | 134.8                           |
| 1 / 50          | 31.07                           | 155.4                           | 55.15                           | 47.34                           | 2.845                           | 98.1                            |
| Blot 4          |                                 |                                 |                                 |                                 |                                 |                                 |
| 1 / 5           | 287.32                          | 143.66                          | 51.00                           | 43.78                           | 2.631                           | 90.7                            |
| 1 / 25          | 79.42                           | 198.54                          | 70.48                           | 60.50                           | 3.636                           | 125.4                           |
| 1 / 50          | 54.85                           | 274.26                          | 97.36                           | 83.57                           | 5.023                           | 173.2                           |
| Blot 5          |                                 |                                 |                                 |                                 |                                 |                                 |
| 1 / 5           | 233.25                          | 116.62                          | 41.40                           | 35.54                           | 2.136                           | 73.7                            |
| 1 / 25          | 88.57                           | 221.43                          | 78.61                           | 67.47                           | 4.055                           | 139.8                           |
| 1 / 50          | 35.04                           | 175.20                          | 62.20                           | 53.39                           | 3.209                           | 110.7                           |
| Mean (n=14)     |                                 |                                 |                                 |                                 | 52.97                           | 3.18                            | 109.8                           |
| Standard Deviation (n=14) |                                 |                                 |                                 |                                 | 6.96                            | 0.42                            | 14.4                           |
Table 5.S1, Continued: Raw data and calculations of V_{H} intrabody expression in *Pichia pastoris* transformants derived from quantitative Western blot analysis (Figure 6.3) and as summarized in Table 6.2.

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>Total V_{H} Lysis* (ng)</th>
<th>V_{H}, 1 mL cell (^1) (µg·mL(^{-1}))</th>
<th>V_{H}, 1 mL pellet (^{††}) (µg·mL(^{-1}))</th>
<th>Calculated Intracellular V_{H} Expression (fg·cell(^{-1})) (^{‡})</th>
<th>(aMol·cell(^{-1})) (^{‡‡})</th>
<th>µMol·L(^{-1}) (^{☺})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blot 1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1 / 5</td>
<td>137.9</td>
<td>68.95</td>
<td>24.48</td>
<td>26.46</td>
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</tr>
<tr>
<td>1 / 5</td>
<td>179.8</td>
<td>89.9</td>
<td>31.91</td>
<td>34.50</td>
<td>2.032</td>
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<td>35.24</td>
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<td>43.11</td>
<td>46.61</td>
<td>2.746</td>
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<tr>
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<td>15.54</td>
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<td>29.82</td>
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<td>49.23</td>
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<tr>
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<td>28.10</td>
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<td>13.92</td>
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<td>Standard Deviation (n=12)</td>
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<td></td>
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<td></td>
<td>2.30</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Calculations, Table 5.S1:

* Total V_{H} Lysis (ng) of diluted V_{H} samples from each assay derived from regression equation analysis based on a five point dilution series (300, 150, 75.0, 37.5, 18.75 ng·lane\(^{-1}\)) of a reference V_{H} fragment (as described in EXPERIMENTAL PROCEDURES).

\[ x = \text{Calculated mass of V}_{H} \text{ in cell lysate (ng)}. \]

\[ y = \text{Measured densometric value of unknown sample (densometric units)}. \]

- **Blot 1**: \[ x = [(y - 3.292) ÷ 0.3387], R^2 = 0.9555 \]
- **Blot 2**: \[ x = [(y + 1.8174) ÷ 0.0913], R^2 = 0.9815 \]
- **Blot 3**: \[ x = [(y + 3.700) ÷ 0.5600], R^2 = 0.9902 \]
- **Blot 4**: \[ x = [(y + 17.561) ÷ 0.3949], R^2 =0.9856 \]
- **Blot 5**: \[ x = [(y + 2.9104) ÷ 0.3456], R^2 =0.9975 \]

\[ V_{H} \text{ from 1 mL Cell Lysis (µg · mL}^{-1}\) = \left[ \frac{\text{Est. } V_{H} \text{ Quantity (ng)}}{\text{Total Vol. Cell Pellet Loaded (µL)}} \right] \times \frac{1,000 \text{ (µL·mL}^{-1}\)}{1,000 \text{ (ng·µg}^{-1}\)}} \]

\[ V_{H} \text{ from 1 mL Cell Pellet (µg · mL}^{-1}\) = \frac{V_{H} \text{ from 1 mL Cell Lysis (µg)}}{\text{Lysis Volume per 1 mL Cell Culture (µL)}} \]
Calculations, Table 5.S1 (Continued):

\[ V_{\text{H}} \text{ per cell (fg·cell}^{-1}) = \left( \frac{V_{\text{H}} \text{ from 1 mL Cell Pellet (µg·mL}^{-1})}{P. \text{pastoris cell density (# cells· 1mL culture}^{-1})} \right) \times \left( \frac{1.0 \times 10^9 \text{ fg}}{1.0 \mu g} \right) \]

\[ V_{\text{H}} \text{ per cell (aMol·cell}^{-1}) = \left( \frac{\text{Intracellular } V_{\text{H}} \text{ per cell (fg·cell}^{-1})}{V_{\text{H}} \text{ MW (fg·fMol}^{-1})} \right) \times \left( \frac{1.0 \times 10^3 \text{ aMol}}{1.0 \text{ fMol}} \right) \]

\[ V_{\text{H}} \text{ Concentration within } P. \text{pastoris cytosol} = \left( \frac{V_{\text{H}} \text{ per cell (a Mol · cell}^{-1})}{29 \text{ femto L · cell}^{-1}} \right) \]

Constants, Table 5.S1:
- Total Volume of Cell Pellet Loaded for 1 / 5; 1/25 and 1/50 dilutions = 2.0; 0.4 and 0.2 µL, resp.
- Lysis Volume per 1.0 mL Cell Culture = 355 µL
- Mean OD\textsubscript{600} of 1.0 mL P. \textit{pastoris} culture for Cytotoxicity Assay-1 (Figure 5.4) = 21.5
- Mean OD\textsubscript{600} of 1.0 mL P. \textit{pastoris} culture for Cytotoxicity Assay-2 (Figure 5.5) = 23.3
- Mean OD\textsubscript{600} of 1.0 mL P. \textit{pastoris} culture for Cytotoxicity Assay-3 (Figure 5.6) = 18.5
- For \textit{Pichia pastoris}, Volume of one cell = 29 femtoliters (2.9 x 10\textsuperscript{-14} Liters)

Therefore,
- Mean \textit{P. pastoris} cell density for Cytotoxicity Assay-1 = (5 x 10\textsuperscript{7} cells·mL\textsuperscript{-1}) X 21.5 = 1.075 x 10\textsuperscript{9}
- Mean \textit{P. pastoris} cell density for Cytotoxicity Assay-2 = (5 x 10\textsuperscript{7} cells·mL\textsuperscript{-1}) X 23.3 = 1.165 x 10\textsuperscript{9}
- Mean \textit{P. pastoris} cell density for Cytotoxicity Assay-3 = (5 x 10\textsuperscript{7} cells·mL\textsuperscript{-1}) X 18.5 = 9.250 x 10\textsuperscript{8}

Molecular Weights (www.expasy.ch/)
- NAT-267 (treatment) \( V_{\text{H}} \text{H} = 16637.3 \text{ Da (fg · fMol}^{-1}) \)
- B-24 (control) \( V_{\text{H}} \text{H} = 16975.6 \text{ Da (fg · fMol}^{-1}) \)
Table 5.S2: HPLC retention times of trichothecene metabolites evaluated after 24 h of growth of *P. pastoris* transformants cultured in YPD media supplemented with 100 µg·mL⁻¹ of highly-purified DON. Transformation of DON (5.750 minutes) to 15-AcDON (9.625 minutes) within supernatant (S/N), cell lysate (LYS), and cell pellet debris (DEBS) shown in 30-minute, 16-h and duplicate 24-h samples of NAT-267 V₁H and pPICZB empty vector transformants.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Peaks (Retention time, min)</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DON</td>
<td>15-AcDON</td>
<td></td>
</tr>
<tr>
<td><strong>Supernatant (S/N) Fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAT-267 V₁H, S/N, 30min.</td>
<td>5.850</td>
<td>H</td>
<td>9.542</td>
<td>NP</td>
</tr>
<tr>
<td>NAT-267 V₁H, S/N, 16h</td>
<td>5.825</td>
<td>H</td>
<td>9.517</td>
<td>H</td>
</tr>
<tr>
<td>NAT-267 V₁H, S/N, 24h, Rep 1</td>
<td>5.833</td>
<td>VH</td>
<td>9.533</td>
<td>VH</td>
</tr>
<tr>
<td>pPICZB (empty vector), S/N, 30min.</td>
<td>5.975</td>
<td>H</td>
<td>9.542</td>
<td>L</td>
</tr>
<tr>
<td>pPICZB (empty vector), S/N, 16h</td>
<td>5.817</td>
<td>VH</td>
<td>9.525</td>
<td>VH</td>
</tr>
<tr>
<td>pPICZB (empty vector), S/N, 24h, Rep 1</td>
<td>5.825</td>
<td>VH</td>
<td>9.542</td>
<td>H</td>
</tr>
<tr>
<td>pPICZB (empty vector), S/N, 24h, Rep 2</td>
<td>5.825</td>
<td>VH</td>
<td>9.533</td>
<td>H</td>
</tr>
<tr>
<td><strong>Cell Lysate (LYS) Fraction</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAT-267 V₁H, LYS, 24h, Rep 1</td>
<td>5.858</td>
<td>M</td>
<td>9.575</td>
<td>M</td>
</tr>
<tr>
<td>NAT-267 V₁H, LYS, 24h, Rep 2</td>
<td>5.875</td>
<td>H</td>
<td>9.567</td>
<td>M</td>
</tr>
<tr>
<td>pPICZB (empty vector), LYS, 24h, Rep 1</td>
<td>5.817</td>
<td>M</td>
<td>9.542</td>
<td>M</td>
</tr>
<tr>
<td>pPICZB (empty vector), LYS, 24h, Rep 2</td>
<td>5.817</td>
<td>M</td>
<td>9.525</td>
<td>L</td>
</tr>
<tr>
<td><strong>Cell Debris (DEBS) Fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAT-267 V₁H, DEBS, 24h</td>
<td>5.550</td>
<td>NP</td>
<td>9.567</td>
<td>NP</td>
</tr>
<tr>
<td>pPICZB (empty vector), DEBS, 24h</td>
<td>5.855</td>
<td>NP</td>
<td>9.525</td>
<td>NP</td>
</tr>
</tbody>
</table>

- No peak (NP) observed
- Low (L) = very small or no peak observed.
- Medium (M) = medium or measurable peak observed.
- High (H) = large peak observed.
- Very High (VH) = very large peak observed.
6 CONCLUSIONS

The research and results summarized within this thesis reaffirms the vast potential of recombinant antibody engineering as a discipline with wide ranging applications. Our specific goal was to develop a rAb fragment with binding affinity to DON, a key mycotoxin produced during FHB pathogenesis. This was a proof of concept to apply a novel technology platform commonly used in pharmaceutical research toward an untested discipline, plant pathology. Our hypothesis was based on the notion that expression of an engineered rAb fragment within a model eukaryotic test system could bind a target mycotoxin and inherently limit in situ cytotoxicity to provide enhanced tolerance to FHB pathogenesis. Although the results of this dissertation are well summarized within Chapters # 4 and # 5, the following provides an overall summary of key conclusions and outlines future research interests and potential technological applications.

Immunization of a llama with 15-DON-BSA conjugate resulted in a robust polyclonal response to the hapten complex (i.e., DON, 15-AcDON, 3-AcDON). A hyper-immunized, phage-display V_H library was successfully created from peripheral blood lymphocytes based on standard procedures (Ghahroudi et al., 1997). A single dominant V_H clone known as ‘NAT-267’ was selected after four rounds of library panning against 15-DON-OVA conjugate. Both NAT-267 V_H monomer and pentamer demonstrated absolute binding specificity to 15-AcDON. NAT-267 V_H nucleotide sequence results (GenBank: EU676170.1 http://lifesciencedb.jp/ddbj/ff_view.cgi?accession=EU676170) confirmed that Ag specificity was generated by somatic hypermutations within the CDR regions. The NAT-267 V_H fragment was highly specific to 15-AcDON as it did not
recognize structurally similar trichothecenes, particularly DON and 3-AcDON. We attributed the exquisite specificity of NAT-267 V_{H}H to the fact that both the immunogen and panning mycotoxin protein conjugates (BSA and OVA, respectively) were linked to DON at carbon 15 using a common hemi-succinate linker.

The methylotrophic yeast *Pichia pastoris* was selected as a model eukaryotic test system based on expected sensitivity to 15-AcDON and well-established capacity to produce well-characterized quantities of functional protein. The *P. pastoris* system was a convenient and cost-effective system to generate reproducible data to assess the ability of intracellular antibodies (intrabodies) to modulate mycotoxin-specific cytotoxicity as a precursor to transformation of higher order plants. Mycotoxin-mediated cytotoxicity was assessed by continuous measurement of cellular growth in real time which allowed for precise measurements of cytotoxicity during initial exposure to each ribotoxin treatment. At equivalent doses, 15-AcDON was significantly more toxic to wild-type *P. pastoris* than was DON which, in turn, was more toxic than 3-AcDON. The order of trichothecene toxicity established within our work aligned with results previously published in other species of yeast and model plant systems. Our observation of no measureable cytotoxicity of 3-AcDON was in agreement with previous studies which demonstrated that the addition of an acetyl ester group at C-3 significantly limits DON cytotoxicity. Intracellular expression of toxin-specific V_{H}H within *P. pastoris* conveyed significant (p = 0.01) resistance to 15-AcDON cytotoxicity at doses ranging from 20 to 100 µg·mL^{-1}. A key limitation of NAT-267 V_{H}H efficacy was that rAb binding to 15-AcDON was of low affinity. The specific limitation was that the Ab-Ag complex most
likely had a short ‘residence time’ which limited $V_H$H efficacy, especially at higher doses of 15-AcDON. Furthermore, we documented a biochemical transformation of DON to 15-AcDON which explained significant attenuation to the phytotoxic effects of DON at 100 and 200 $\mu$g·mL$^{-1}$. This “proof of concept” model established in this work suggests that *in planta* $V_H$H expression may lead to enhanced tolerance to mycotoxins and thereby serve as a novel tool to help reduce the impact of *Fusarium* infection of commercial agricultural crops. Furthermore, based on these results, we accept our hypothesis in affirming that the expression of a recombinant $V_H$H antibody fragment within a model eukaryotic test system can limit mycotoxin-specific cytotoxicity. However, we reiterate that applications of mycotoxin-specific rAb fragments would be most effectively applied as a tool to complement (and hopefully enable) other means of enhanced FHB resistance in higher order plants.

We recommend that future experiments and next steps be focused on the development of $V_H$H rAbs with improved dissociation constants ($K_D$) to ensure a longer residence time supported by stronger association between $V_H$H and the target ligand for improved efficacy (Copeland et al., 2006). It would also be of interest to develop and test novel $V_H$H fragments for various other trichothecenes (e.g., neosolaniol, diacetoxyscripenol, T-2 toxin, etc.) and perhaps other classes of mycotoxin (e.g., fumonisins, aflatoxins, etc.). A logical subsequent application would be constitutive *in planta* expression of optimized mycotoxin-specific $V_H$H fragments, possibly with catalytic activity, to bind and deactivate/degrade mycotoxins during critical initial periods of *Fusarium* pathogenesis.


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