

ISOLATION AND STRUCTURAL CHARACTERIZATION OF THE ACTIVE MOLECULE
FROM SIREX NOCTILIO WOODWASP VENOM INDUCING PRIMARY
PHYSIOLOGICAL SYMPTOMS IN ATTACKED PINE SPECIES

by

JOHN MICHAEL BORDEAUX

(Under the Direction of Jeffrey F. D. Dean)

ABSTRACT

Sirex noctilio F., a non-native, invasive woodwasp recently introduced into North America, attacks and kills living pines, including economically important species. *S. noctilio* introduces phytotoxic venom and a pathogenic basidiomycete, *Amylostereum areolatum*, into trees during oviposition. *S. noctilio* venom alone induces pine needle chlorosis and abscission, phloem collapse, altered respiration, loss of carbohydrate translocation, and reduced growth rate.

A 26,496-feature loblolly pine cDNA microarray was used to identify two genes useful as biomarkers in qRT-PCR bioassays for pine response to venom exposure, PR4 and TLP. Expression of both was strongly induced in response to venom, while expression of an apparent actin gene (ACT1) was stable in response to the venom.

The *S. noctilio* venom gland transcriptome was assembled from >260 million Illumina HiSeq reads using a minimum length cutoff for assemblies of 100 nucleotides. This dataset is the first transcriptome-scale resource available for a basal-order species of Hymenoptera. Of the 68,887 transcripts generated from this dataset, 24,471 (35%) returned annotations with E- value $\leq 1 \times 10^{-10}$. No annotations were returned for the seven most abundant transcripts. The single

most abundant transcript present encodes the molecule responsible for initial wilt and gene responses in pines. The eighth most highly-expressed sequence recovered was a transcript encoding a putative laccase-like multicopper oxidase.

The bioassay above was utilized to isolate from *S. noctilio* venom an 11-amino acid peptide (SEGPROGTRKP) of 1850 Da. This peptide, noctilisin, induced the wilt phenotype in pine seedling explants. Primary sequence and structure were established using Edman degradation, NMR, and MS-MS. Post-translational modifications include O-glycosylation of serine and threonine, and hydroxylation of the 6th residue (proline). Two identical O-glycans present were characterized as N-acetylgalactose, modified by an O-linked phosphoethanolamine on the glycan C6. Noctilisin activity is glycan-dependent. Wide-scale screening for resistance to woodwasp attack across pine populations now is feasible.

A further RNA-Seq experiment was performed to compare the effects of whole venom, noctilisin alone, and a biologically-inactive, non-glycosylated peptide on sensitive *P. radiata* explants. These results should elucidate suites of genes responding specifically to noctilisin and distinguish them from genes responding to other active factors in whole venom.

INDEX WORDS: bioassay, forest health, glycopeptide, Hymenoptera, LMCO, loblolly, pine, *Pinus radiata*, *Pinus taeda*, woodwasp, *Sirex noctilio*, Symphyta, transcriptome, venom

ISOLATION AND STRUCTURAL CHARACTERIZATION OF THE ACTIVE MOLECULE
FROM SIREX NOCTILIO WOODWASP VENOM INDUCING PRIMARY
PHYSIOLOGICAL SYMPTOMS IN ATTACKED PINE SPECIES

by

JOHN MICHAEL BORDEAUX

Bachelor of Science, University of North Carolina at Chapel Hill, 1992

Master of Science, University of Georgia, 2008

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2014

© 2014

John Michael Bordeaux

All Rights Reserved

ISOLATION AND STRUCTURAL CHARACTERIZATION OF THE ACTIVE MOLECULE
FROM SIREX NOCTILIO WOODWASP VENOM INDUCING PRIMARY
PHYSIOLOGICAL SYMPTOMS IN ATTACKED PINE SPECIES

by

JOHN MICHAEL BORDEAUX

Major Professor: Jeffrey F. D. Dean

Committee: Claiborne V. C. Glover III
Scott E. Gold
Michael R. Strand

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2014

ACKNOWLEDGEMENTS

This work was made possible by the efforts of the following indispensable persons:

Jeff Dean: perspective, tireless guidance and consistent enthusiasm for the endeavor;

Walt Lorenz: critical analysis, hard scientific work, and daily assistance;

Claiborne Glover, Scott Gold, Michael Strand, Kamal Gandhi: rigor, honest criticism, abiding scientific interest, substantial counsel, material support, and friendship;

D.J. Johnson, Majors Badgett, Ron Orlando, John Glushka, Bernard Slippers, Raj

Ayyampalayam, Dave Coyle: scientific collaboration and generous contributions during experimental sampling and analysis;

Ujwal Bagal, Susan Wilde, Dick Daniels, Mark Brown, Scott Merkle, Giselle Dean, Alan

Przybyla, Zac Wood, Nanda Menon, Gwen Hirsch, Martin Kallins, Valeria Coscia, Bruce

Johnson, C.J. Tsai, Eileen Carroll, Jay Shelton, Kris Irwin, Diana Hartle, Amonae Dabbs-Brown,

Joyce Black, Angie Callaway, Kate Dedufour, Judith Willis: advice, interest, support, friendship, encouragement;

Matt Head, David Rutland, Carolyn and Calvin Monroe, Grady and Glenda Bordeaux, Chris and

Ashely Bordeaux, Sheila and Steve Rains, Randy Lindstrom, Dan and Susanne Reeder: family support in word and deed.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iv
LIST OF TABLES	ix
LIST OF FIGURES	xi
CHAPTER	
1 INTRODUCTION	1
1.1 Pine, Economic Importance, and Potential Threats to Pine Health	1
1.2 Siricid Woodwasps	2
1.3 Significant Players in the Woodwasp/Pine Pathosystem.....	2
1.4 Research Objectives.....	2
References.....	5
2 LITERATURE REVIEW: SUSCEPTIBILITY AND RESPONSE OF PINES TO SIREX NOCTILIO	7
2.1 Introduction.....	9
2.2 Factors Contributing to Tree Susceptibility.....	11
2.3 Tree Responses to <i>Sirex noctilio</i> Mucus.....	14
2.4 Tree Responses to <i>Amylostereum areolatum</i>	20
2.5 General Responses to Sirex Attack.....	23
2.6 Reducing Tree Susceptibility.....	24
References.....	29

3	BIOMARKER GENES HIGHLIGHT INTRASPECIFIC AND INTERSPECIFIC VARIATIONS IN THE RESPONSES OF PINUS TAEDA L. AND PINUS RADIATA D. DON TO SIREX NOCTILIO F. ACID GLAND SECRETIONS	42
3.1	Introduction.....	44
3.2	Materials and Methods.....	46
3.3	Results.....	52
3.4	Discussion	56
3.5	Conclusions.....	60
	References.....	71
4	CHARACTERIZATION OF THE VENOM GLAND TRANSCRIPTOME FROM THE WOODWASP, SIREX NOCTILIO F., IDENTIFIES HIGHLY EXPRESSED TRANSCRIPTS ENCODING A LACCASE-LIKE MULTICOPPER OXIDASE AND OTHER PROTEINS THAT MODULATE THE RESPONSE OF PINES TO INSECT ATTACK	78
4.1	Introduction.....	80
4.2	Materials and Methods.....	84
4.3	Results.....	88
4.4	Discussion	95
4.5	Conclusions.....	100
	References.....	114
5	NOCTILISIN, A VENOM GLYCOPEPTIDE, IS THE HEAT-STABLE FACTOR THAT CAUSES NEEDLE WILT AND DEFENSE GENE RESPONSES IN PINES ATTACKED BY THE SIREX NOCTILIO WOODWASP.....	130

5.1	Introduction.....	132
5.2	Experimental Procedures	134
5.3	Results.....	138
5.4	Discussion	142
	References.....	162
6	PINE DIFFERENTIAL EXPRESSION RESPONSES TO NOCTILISIN AND WHOLE SIREX NOCTILIO VENOM	173
6.1	Introduction.....	173
6.2	Materials and Methods.....	175
6.3	Results and Conclusions	176
	References.....	177
7	CONCLUSIONS.....	180
7.1	Noctilisin Initiates the <i>Sirex noctilio</i> Pine Wilt Pathology	180
7.2	Venom: Transcriptome, Proteome, Glycosylation.....	184
7.3	Enhancement of Future Pine Breeding Populations	188
7.4	Concluding Remarks.....	189
	References.....	193

APPENDICES

A	HIGH VARIABILITY IN BIOMARKER GENE RESPONSES TO SIREX NOCTILIO VENOM IN FIELD-GROWN PINES	196
A.1	Introduction.....	197
A.2	Materials and Methods.....	198
A.3	Results.....	199

A.4 Discussion	201
References	208
B PRELIMINARY PROTEOMIC ANALYSIS OF SIREX NOCTILIO VENOM AND CORRELATION WITH VENOM TRANSCRIPTOMIC DATA	210
B.1 Introduction	210
B.2 Materials and Methods	211
B.3 Results	212
B.4 Discussion	215
References	223

LIST OF TABLES

	Page
Table 3.1: Genes identified in <i>P. taeda</i> seedlings showing the greatest fold-expression increases (log ₂ normalized) at 24 and 48 h post-treatment with <i>S. noctilio</i> venom	62
Table 3.2: Gene annotations and primer sequences used in PCR assays.....	63
Table 4.1: The twenty most abundant transcripts from <i>S. noctilio</i> venom gland tissue that returned annotations, excluding ribosomal proteins	103
Table 4.2: The twenty most abundant transcripts from <i>S. noctilio</i> venom gland tissue that did not return annotations.....	104
Table 4.3: Transcripts from <i>S. noctilio</i> venom gland tissue encoding apparent amylases	105
Table 4.4: Transcripts from <i>S. noctilio</i> venom gland tissue encoding apparent secreted esterases	106
Table 4.5: Transcripts from <i>S. noctilio</i> venom gland tissue encoding apparent secreted phenoloxidases	107
Table 4.6: Transcripts from <i>S. noctilio</i> venom gland tissue encoding apparent secreted proteases or peptidases.....	108
Table 4.7: Transcripts from <i>S. noctilio</i> venom gland tissue returning annotations matching plant proteins.....	109
Table 5.1: Proton chemical shifts for noctilisin glycopeptide in D ₂ O at 25 °C.....	148
Table 7.1: <i>S. noctilio</i> venom transcripts coding for galactosyltransferases	191

Table 7.2: Peptide fragments obtained for an apparent peptidyl-GalNAc transferase in <i>S. noctilio</i> woodwasp venom.....	192
Table B.1: Transcriptome sequences with corresponding proteomic hits having relative expression values > 0.2.....	218
Table B.2: Protein abundance correlated with transcript abundance.....	219
Table B.3: Proteomic data correlated with significant transcript abundance	220
Table B.4: Peptide fragments obtained for an apparent LMCO in <i>S. noctilio</i> woodwasp venom.....	221
Table B.5: Peptide fragments obtained for an alpha amylase homolog in <i>S. noctilio</i> woodwasp venom.....	222

LIST OF FIGURES

	Page
Figure 2.1: <i>Sirex noctilio</i> infestations in overstocked pulpwood plantations of <i>P. patula</i> in South Africa (panel A) and <i>P. resinosa</i> in New York, USA (panel B)	26
Figure 2.2: Extent of tree responses to <i>Sirex noctilio</i> attack.....	27
Figure 2.3: Bioassay of <i>S. noctilio</i> venom activity using 4-month-old <i>P. radiata</i> seedlings, cut at soil line, 7 days after exposure.....	28
Figure 3.1: Typical phenotypic responses in pine seedlings treated with <i>S. noctilio</i> venom	64
Figure 3.2: Semi-quantitative PCR analysis of pine gene response to <i>S. noctilio</i> venom	65
Figure 3.3: <i>Pinus taeda</i> gene responses to <i>S. noctilio</i> venom.....	66
Figure 3.4: <i>Pinus radiata</i> gene responses to <i>S. noctilio</i> venom.....	67
Figure 3.5: Gene expression-level differences between pine species in response to woodwasp venom exposure	68
Figure 3.6: Within-species variation in <i>P. radiata</i> biomarker gene response to <i>S. noctilio</i> venom.....	69
Figure 3.7: The effect of proteolysis on induction of biomarker genes by <i>S. noctilio</i> venom.....	70
Figure 4.1: <i>Sirex noctilio</i> venom apparatus	110
Figure 4.2: Zymogram analysis of laccase activity in aqueous extracts of <i>S. noctilio</i> venom glands	111
Figure 4.3: Laccase bootstrapping tree for the two principal putative phenoloxidase transcripts in <i>Sirex noctilio</i> venom tissue	112

Figure 4.4: PCR test for presence of <i>Deladenus</i> sp. in <i>S. noctilio</i> females	113
Figure 5.1: RP-HPLC fractionation of venom 10 kDa pass-through and bioassay evaluation ...	149
Figure 5.2: RP-HPLC fractionation of venom 10 kDa pass-through to single components and bioassay evaluation	150
Figure 5.3: RP-HPLC purification of noctilisin.....	151
Figure 5.4: Molecular mass determination of noctilisin using MALDI-TOF/MS.....	152
Figure 5.5: Noctilisin transcript and amino acid sequence	153
Figure 5.6A: NMR spectral analysis of purified noctilisin.....	155
Figure 5.6B: NMR spectral analysis of noctilisin glycans	156
Figure 5.6C: 1D (¹ H) ³¹ PHMQC spectrum showing signals from ethanolamine protons as well as GalNAc H6 protons coupled to phosphorous	157
Figure 5.7: Noctilisin structure	158
Figure 5.8: Typical pine shoot tip bioassay responses.....	159
Figure 5.9: Dose-dependence of noctilisin bioactivity via qRT-PCR	160
Figure 5.10: Dependence via qRT-PCR of noctilisin bioactivity on glycosyl modification	161
Figure A.1: Cross-sections of Monterey pine (<i>P. radiata</i>) needles exposed to fresh <i>S. noctilio</i> venom, 10X.....	203
Figure A.2: RT-PCR products amplified from seven species of <i>Pinus</i> using primer pairs based on <i>P. taeda</i> genes	204
Figure A.3: Expression levels of biomarker genes in pine needles treated with <i>S. noctilio</i> venom	205
Figure A.4: Apparent biomarker gene expression responses to <i>S. noctilio</i> venom in needles collected on different dates via qRT-PCR	206

Figure A.5: Fold-change in expression of biomarker genes in three pine species responding to *S. noctilio* venom via qRT-PCR.....207

CHAPTER 1

INTRODUCTION

1.1 Pine, Economic Importance, and Potential Threats to Pine Health

Pines belong to the genus *Pinus*, among conifers one of the most widely-used trees for structural timber, reforestation, and soil erosion prevention (1,2). Pines are especially important economic forest tree species in the United States. Loblolly pine (*Pinus taeda* L.) is among pines the most important structural timber species in the U.S., and is also grown on a large scale in Australia, Brazil, China, New Zealand, and South Africa. Pines, particularly North American pines, play an outsized role in the economies of nations in the southern hemisphere. For instance, in Australia, pines comprise 80% of commercial forests, and in New Zealand's commercial forests have been reported as containing 89% radiata pine (*Pinus radiata* D. Don) (3). Radiata pine in particular is grown extensively as an exotic in the southern hemisphere, covering nearly 4 million hectares (2).

In addition to the threat of drought, overcrowding, and other indigenous pests, pines face challenges to their health in the form of invasive insects. While insects are introduced to naïve hosts routinely through interstate and international commerce, the factors affecting invasiveness in their new environments are not yet fully established. An insect species can be invasive because of similarity to an indigenous species (*Sirex noctilio*, the European woodwasp, shares both a lifestyle and many host preferences with North American native woodwasp *Sirex nigricornis*), or because it is sufficiently different from native species to occupy a previously unoccupied niche (*S. noctilio* favors living conifers in good health, whereas *S. nigricornis* prefers

moribund or dying conifers) (4). The emergence of lethal insect-fungal symbioses in recent years and their effects once introduced to naïve hosts has become a topic of particular concern (5).

1.2 Siricid Woodwasps

Woodwasps are phytophagous members of a basal order of the insect order Hymenoptera. Woodwasps of the genus *Sirex* exist as native species in North America, where they oviposit into dead or dying conifers. When *Sirex noctilio* has been introduced into southern hemisphere forests populated with North American pines, the insects attack these pines preferentially, causing massive damage. *Sirex noctilio*, the European horntail woodwasp, was recently introduced into North America. It is a concern that this insect may pose a similar threat to pines in North America if its establishment is not limited.

1.3 Significant Players in the Woodwasp/Pine Pathosystem

Sirex noctilio induced pathology in pines is at least a tritrophic system, as the woodwasps vector a white-rot fungal symbiont, *Amylostereum areolatum* (6,7). Additionally, male and female *S. noctilio* individuals may harbor nematodes (*Deladenus* spp.) which may take a free-living, *A. areolatum* fungus-feeding form, or instead invade the gonads of the woodwasp in another form, causing sterility (8).

1.4 Research Objectives

To completely understand the pathology in pine that follows woodwasp attack, it is necessary to isolate the role of key events and players. The principal focus of this work has been to elucidate the role of the woodwasp's venom secretion in initiation of the primary symptoms associated with woodwasp attack.

1.4.1 Isolation and characterization of pine wilt-inducing factor (noctilisin) from *S. noctilio* venom

It was established by earlier investigators that venom is the causative agent for the initial suite of symptoms following woodwasp attack, including chlorosis and abscission of needles, collapse of phloem, loss of photosynthate translocation, reduced growth rate, and altered respiration (9-11). Partially-purified fractions of the venom have been shown to induce at least some of these symptoms (12). Hymenopteran venoms are highly-complex mixtures of enzymes, small peptides, and other small molecules. To understand the symptoms of the pathology, it is necessary to isolate the causative agent from this mixture.

1.4.2 Develop a database for *S. noctilio* venom tissue transcriptome using whole transcriptome shotgun sequencing (RNA-seq) to help identify the gene encoding noctilisin

Evidence produced here and elsewhere indicated the nature of the factor causing pine wilt is at least partially proteinaceous (Chapter 3, 5 this work) (13). Given that the molecule is produced in the venom gland of the woodwasp, it is most likely that a profile of the entire transcriptome of the venom tissue should reveal one or more transcripts encoding a proteinaceous factor causing wilt symptoms.

1.4.3 Characterize the transcriptome profile of pine genes responsive to *S. noctilio* venom to identify additional active factors in the woodwasp secretions

Evidence is presented here that the venom contains a factor that exerts its effects in the photosynthetic tissue (needles) of conifers. However, venom being a complex mixture, it is likely that there are effects exerted at the site of oviposition by venom components other than the translocated wilt factor. Distinguishing the effects of the translocated wilt factor from those of whole venom may be accomplished subtractively once the wilt factor is isolated from whole

venom. If these effects are exerted at the level of gene expression, they may be measured over time via whole-transcriptome shotgun sequencing of pine tissue treated with whole venom versus isolated wilt factor. This study documents the initial experiments performed to distinguish the effects of wilt factor from those of whole venom; the data have been generated and analysis is underway. The results and conclusions will appear in a later manuscript.

References

1. Mackay, J., Dean, J. F. D., Plomion, C., Peterson, D. G., Canovas, F. M., Pavy, N., Ingvarsson, P. K., Savolainen, O., Guevara, M. A., Fluch, S., Vinceti, B., Abarca, D., Diaz-Sala, C., and Cervera, M. T. (2012) Towards decoding the conifer giga-genome. *Plant Molecular Biology* **80**, 555-569
2. Praciak, A., Pasiecznik, N., Sheil, D., van Heist, M., Sasses, M., Correia, C. S., Dixon, C., Fyson, G. E., Rushforth, K., and Teeling, C. (2013) The CABI Encyclopedia of Forest Trees. CAB International, Boston
3. Webster, J., and Mota, M. (2008) Pine Wilt Disease: Global Issues, Trade, and Economic Impact. in *Pine wilt disease: a worldwide threat to forest ecosystems* (Vieira, P., and Mota, M. eds.), Springer, Dordrecht. pp 1-3
4. Lieutier, F. (2006) Changing Forest Communities: Role of Tree Resistance to Insects in Insect Invasions and Tree Introductions. in *Invasive Forest Insects, Introduced Forest Trees, and Altered Ecosystems*, Springer Netherlands. pp 15-51
5. Hulcr, J., and Dunn, R. R. (2011) The sudden emergence of pathogenicity in insect-fungus symbioses threatens naive forest ecosystems. *Proc. R. Soc. B-Biol. Sci.* **278**, 2866-2873
6. Coyle, D. R., and Gandhi, K. J. K. (2012) The ecology, behavior, and biological control potential of hymenopteran parasitoids of woodwasps (Hymenoptera: Siricidae) in North America. *Environmental Entomology* **41**, 731-749
7. Talbot, P. H. B. (1977) *Sirex-Amylostereum-Pinus* association. *Annual Review of Phytopathology* **15**, 41-54

8. Slippers, B., Hurley, B. P., Mlonyeni, X. O., de Groot, P., and Wingfield, M. J. (2012) Factors Affecting the Efficacy of *Deladenus siricidicola* in Biological Control Systems. *Sirex Woodwasp and Its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest*, 119-133
9. Coutts, M. P. (1968) Rapid physiological change in *Pinus radiata* following attack by *Sirex noctilio* and its associated fungus, *Amylostereum* sp. *Aust. J. Sci.* **30**, 275-277
10. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .2. Effects of *S. noctilio* mucus. *Aust. J. Biol. Sci.* **22**, 1153-1161
11. Fong, L. K., and Crowden, R. K. (1973) Physiological effects of mucus from wood wasp, *Sirex noctilio* F., on foliage of *Pinus radiata* D. Don. *Aust. J. Biol. Sci.* **26**, 365-378
12. Bordeaux, J. M., Lorenz, W. W., and Dean, J. F. D. (2012) Biomarker genes highlight intraspecific and interspecific variations in the responses of *Pinus taeda* L. and *Pinus radiata* D. Don to *Sirex noctilio* F. acid gland secretions. *Tree Physiol.* **32**, 1302-1312
13. Wong, L. K., and Crowden, R. K. (1976) Preliminary studies on mucus secretion of wood wasp, *Sirex noctilio* F .1. Physicochemical and biochemical properties. *Aust. J. Biol. Sci.* **29**, 21-32

CHAPTER 2

LITERATURE REVIEW:

SUSCEPTIBILITY AND RESPONSE OF PINES TO SIREX NOCTILIO¹

¹ Bordeaux, J.M. and J.F.D. Dean, 2012, The *Sirex* Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest. Springer, Ch. 3, 31-50. Reprinted here with permission of the publisher.

Abstract

Sirex noctilio uses an unusual, two-component approach to kill pine trees and provide a source of nutrition for its developing larvae. A substantial amount of research was undertaken in the 1960s and 70s to examine the mechanisms by which these two components – a mucus produced in the woodwasp acid- (venom) gland, and a pathogenic fungus, *Amylostereum areolatum*, both of which are introduced into trees during oviposition – contribute to the death of trees. This chapter reviews the effects that the woodwasp mucus was shown to have when introduced to trees in the absence of the fungus, including resinosis, premature senescence and abscission of needles, and collapse of parenchymal cells with consequent loss of photosynthate translocation, as well as changes in respiration, and reduced growth rates. The effects seen in trees that were artificially inoculated with *A. areolatum*, including increased ethylene production and the induction of polyphenol biosynthesis, are also reviewed. Also discussed are the roles played by tree health and environmental stresses in determining overall susceptibility of host trees to attack by *S. noctilio*. These past observations are within the context of our current understanding of plant defense responses to attack by insects and pathogens, and a number of promising areas for future research are highlighted.

2.1 Introduction

As discussed elsewhere in this book, *Sirex noctilio* F. (Hymenoptera: Siricidae) is a woodwasp native to Europe and northern Africa (1). A member of the Symphyta (Sawfly) suborder of the Hymenoptera (2), *S. noctilio* attacks a broad range of pine species, laying eggs in the sapwood of host trees through holes drilled in the bark (3). During oviposition, *S. noctilio* inoculates the host tree with a symbiotic fungal pathogen, *Amylostereum areolatum* (Fr.) Boidon, that not only kills the tree, but is also the primary food source for developing wasp larvae (4). Along with the fungus, the wasp also injects a so-called ‘mucus’ that affects tree responses and assists the fungus in establishing a productive infection (5,6). Thus, we must dissect the recognition and response mechanisms of all three organisms if we are to fully understand the infection process.

S. noctilio is not typically considered a significant threat to trees in its native range where the forest species have evolved in concert with this pest and attacks are usually limited to suppressed and dying trees (7-9). Woodwasps in the Siricidae family, including *Sirex*, are successful on conifers generally, but *S. noctilio* shows a marked preference for pines and is the only Siricid in its range that attacks living trees (10). In its native range (Europe, Turkey, and North Africa) primary host species include Turkish (*Pinus brutia* Ten.), Canary Island (*P. canariensis* Smith), Aleppo (*P. halapensis* Mill.), Austrian (*P. nigra* Arn.), maritime (*P. pinaster* Ait.), stone (*P. pinea* L.), and Scots pines (*P. sylvestris* L.) (1). However, where *Sirex* populations have become established in the southern hemisphere, it has proven a devastating problem for commercial pine plantations. These commercial forests are frequently planted with North American species, such as radiata (*P. radiata* D. Don) and loblolly pines (*P. taeda* L.), that have not previously been exposed to *S. noctilio* and in environments that do not contain other

Siricids or their associated predators. Under these conditions *S. noctilio* populations can rapidly expand to the point where the woodwasps attack and overwhelm healthy trees (1,11).

Consequently, there is great concern about the potential damage this pest may bring to North American forests now that it has established itself in the region around Lake Ontario (12). Within the current boundaries of this first North American infestation, eastern white pine (*P. strobus* L.) and red pine (*P. resinosa* Ait.) appear to be the species being hardest hit, although Scots pines in overstocked and abandoned Christmas tree plantations are also suffering heavy mortality (12).

Other known North American pine hosts for *S. noctilio* include knobcone (*P. attenuate* Lemm.), jack (*P. banksiana* Lamb.), Caribbean (*P. caribaea* Mor.), lodgepole (*P. contorta* Dougl.), shortleaf (*P. echinata* Mill.), slash (*P. elliotii* Engl.), Jeffrey (*P. jeffreyi* Balf.), Bishop (*P. muricata* D. Don), longleaf (*P. palustris* Mill.), Mexican weeping (*P. patula* Schiede ex Schtdl. & Cham.), and ponderosa pines (*P. ponderosa* Dougl. ex Laws.) (11). The economic impact from *Sirex* outbreaks can be serious when they are left unchecked, and a recent estimate suggested that such an outbreak in U. S. forests might lead to losses as high as \$3 - \$17 billion in the affected areas (13).

In this chapter we review what is known about pine susceptibility and response to attack by *S. noctilio*. Our discussion begins with coverage of the conditions predisposing trees to *Sirex* attack, and is followed by separate descriptions of tree responses to woodwasp mucus, responses to the fungal pathogen, and responses for which the inducer remains unclear. We conclude with prescriptions for reducing the severity of *Sirex* attacks as well as a discussion of areas needing further research.

2.2 Factors Contributing to Tree Susceptibility

Pines are predisposed to attack by *Sirex noctilio* by a wide variety of biotic and abiotic factors. Across their native range, *Sirex* females prefer suppressed or sickly trees, which have not been favored with sufficient light, nutrients, water, or other resources (14,15). Tree age and size at the time of attack also affect susceptibility. Smaller-diameter trees are favored by *S. noctilio* females, although larger trees may be attacked during later years of an outbreak (16). Large and healthy dominant trees are least favored for ovipositing, and such trees are most likely to survive attack (4,17). Environmental stresses, particularly drought, trigger stress responses in the trees that act as signals for *S. noctilio* attack (1). An outbreak following a severe drought caused losses of 25-33% over 300,000 acres of *P. radiata* plantations New Zealand between 1946 and 1951 (18). More recently, the implications of declining tree vigor in the face of drought conditions and climate change for expansion of populations of *S. noctilio* and other wood-boring pests was examined for Scots pine forests in Switzerland (19). Overstocking and poor silvicultural practices that lead to root exposure, flooding, or wounding of standing trees, as well as poor sanitation practices and monocultures in plantations, can contribute to create conditions conducive to *Sirex* outbreaks (1).

Phytophagous insects are often attracted by wounding of potential hosts (20,21). *S. noctilio* females are attracted immediately to trees that have been felled or injured by events such as fire, windthrow, or lightning, and the attraction seems primarily motivated by monoterpenes and possibly other volatiles released from injured tree stems (14). Trapping methods to monitor *S. noctilio* populations take advantage of this phenomenon, and the currently recommended lure to attract female wasps to artificial traps is a 70:30 mixture of α - and β -pinene, respectively (22). However, evidence continues to suggest that additional volatile compounds may be involved in

signaling since girdled and/or herbicide-treated trap trees (23-27) nearly always outperform artificial traps baited with the recommended mixed-pinene lure (V. Mastro, *personal communication*).

Presumably, forest pests that reduce tree vigor and suppress growth, such as defoliating insects, bark beetles, or root nematodes, would make pines more susceptible to attack by *S. noctilio*. Certainly, pines that have been weakened from prior attacks by *S. noctilio* are more attractive and susceptible to subsequent Sirex attacks, and this is the primary means by which once-healthy and dominant trees are eventually killed during large Sirex outbreaks (14,28-30). Although synergisms between Sirex and other pests is an area of research that has not received much attention, it was reported that trees infected by *Heterobasidion annosum* (Fr.) Bref. and consequently suffering annosus root-rot were weakened and vulnerable to Sirex attack (1). In contrast, there is anecdotal evidence that blue-stain fungi and *A. areolatum* are mutually antagonistic to the growth of one another (17,31).

Once a *S. noctilio* female selects a promising tree for oviposition she drills a series of test bores and, using a sensory apparatus in her ovipositor, makes a determination of whether to deposit eggs based on the perceived moisture content and resin pressure of the wood (17,29,31). Drought stress typically reduces resin pressure in conifers, and it can also affect resin composition and subsequent resistance to insect pests (32-34). When tree phloem sap is under high osmotic pressure, woodwasp females reject the tree (3), ostensibly because the resin, which is lethal to Sirex eggs, is more likely to flow into and fill egg galleries in such trees (35). Small, abundant resin droplets on tree stems are often the first symptom noticed in pines under attack from *S. noctilio* (12,29,36). Streaming of resin on the trunks of newly attacked healthy trees has been described as a “thin dribble” (18) and was correlated with resistance, while beading of resin

as single droplets was more often the case on trees that were likely to succumb (37). Helpful illustrations of resin beading and streaming are to be found in the report by Titze & Stahl (36).

As trees weaken and resin pressures drop due to multiple woodwasp attacks, females drill fewer single-bore test holes and more multi-bore galleries into which they lay their eggs (29,38). The galleries typically comprise 2-4 tunnels (0.5 mm diameter) drilled into the sapwood 5-9 mm below the cambium (15,39,40). All of the tunnels drilled by *Sirex* females receive mucus and inoculation with arthrospores of *A. areolatum*, but fewer than half the tunnels receive an egg (15,35,39). Although resin is produced constitutively in conifer tissues, it may also be induced by exposure to fungal pathogens (41). Thus, resinosis was induced in *P. radiata* by manual inoculation with *A. areolatum* from purified cultures (36).

The drilling of tunnels through the inner bark (phloem) and into the sapwood (xylem) during oviposition constitutes a wounding event that elicits responses from pine that share many of the attributes common to wound responses in other plants (42-44). When bark over oviposition holes is removed, darkly stained, necrotic lesions are sometimes noted in the cambium (29). More commonly, a vertically elongated lens-shaped area of wood stained reddish-brown discoloration quickly becomes apparent around the site of oviposition (45). The discoloration likely stems from polyphenolic compounds induced in response to wounding or the fungal pathogen, but may also be associated with a polyphenoloxidase activity in the wasp mucus (6). The production of resin and polyphenolic compounds associated with resistance to *S. noctilio* was shown to vary between dominant and suppressed trees (35,46). Kopke et al. (47) recently demonstrated that in response to oviposition by sawflies in its needles, Scots pine alters the expression of sesquiterpene synthase genes so as to release volatile compounds that attract sawfly parasitoids. Recent studies have shown heritable variation in the quantity and quality of

secondary compounds produced by pines and other conifers responding to wounding and various pests (48-51). These and similar observations suggest that increased resistance to *S. noctilio* may be achieved in pines through directed breeding, but a substantial amount of work remains to identify the specific genes and alleles that require selection to achieve this goal.

Ethylene is a gaseous hormone that plays a wide variety of roles in plants, including regulation plant defense responses, such as the production of chitinase and the lignification of plant cell walls (52,53). Dominant *P. radiata* trees attacked by *S. noctilio* were able to increase production of ethylene by more than an order of magnitude compared to suppressed trees, and production of ethylene was more than three times greater in trees attacked by *Sirex* than in control trees damaged mechanically (54). Recent studies make clear that ethylene produced in response to wounding or pathogens leads to increased production of resin ducts in conifers and this consequently enhances resin flow, which is the primary line of defense against insects (55,56). It seems likely that the higher levels of ethylene produced in dominant trees may be responsible, at least in part, for the increased resin flows that are also associated with the resistance of pines to attack by *S. noctilio*.

2.3 Tree Responses to *Sirex noctilio* Mucus

In an important series of studies fundamental to our current understanding of the interaction between *S. noctilio* and its hosts, Coutts (5,6,46) demonstrated that the mucus and the fungus injected into the tree during oviposition were responsible for different symptoms in the overall pathology. Neither the mucus nor the fungus alone was sufficient to kill trees, but the combination was lethal. Thus, the mucus was characterized as a ‘conditioning’ agent necessary for establishing an active and overwhelming infection of the host by *A. areolatum* (6). It was also

noted that in order for it to cause wilting and senescence of needles in the crown of trees – among the most obvious early symptoms of Sirex attack – the mucus must contain elements that can quickly and easily diffuse through the transpiration stream.

S. noctilio produces its mucus in an accessory gland that is a cognate to the acid- or venom glands found in stinging wasps and bees (Aculeata). The mucus, a clear, sticky and highly viscous material, is stored in a reservoir attached through ducts to the woodwasp reproductive tract near the base of the ovipositor (57). An early attempt at biochemical characterization identified non-sulfated acid mucopolysaccharides as a major component of Sirex mucus (58). The study also reported amylase, esterase, protease and phenoloxidase activities in the mucus. Experiments to isolate and characterize the diffusible active factor(s) responsible for needle wilt and senescence noted that a heat-stable (autoclavable) component dissociated from the mucus in water, initially with an apparent molecular mass of 60-100 kDa. This material subsequently dissociated into smaller 2-6 kDa subunits with no reduction in physiological activity. These characteristics are consistent with a small, bioactive peptide, but this has not been tested using appropriate procedures, such as protease digestion or exposure to amino acid-modifying agents.

Arthropod venoms are a rich source of biologically active peptides that have provided promising leads for a variety of biotechnological applications (59). Mastoparan, an antimicrobial peptide commonly found in stinging wasp venoms, is widely utilized to study trimeric G-protein signaling in animal cells (60); however, mastoparan has also been shown to activate mitogen-activated protein (MAP) kinase signaling cascades in plants (61). Among other plant responses, MAP kinase cascades have been implicated in the senescence of various plant tissues (62). Whether mastoparan or a related peptide is present in *S. noctilio* mucus where it might play a role in inducing pine needle senescence is as yet unknown. Kuhn-Nentwig (63) has nicely

described how the mélange of peptides, enzymes and low molecular weight compounds contribute to the dual function of arthropod venoms as both antimicrobial agents and toxins that alter host physiology. Kellner (64) has noted the importance of antimicrobial agents for protecting insect eggs against pathogens, which suggests that examination of *Sirex mucus* for antimicrobial activities might also be a productive line of inquiry. It seems a reasonable evolutionary progression that antimicrobial mucus, originally deposited with eggs to protect them from pathogens, might undergo natural selection for phytotoxic activities that could contribute to the establishment of a fungal food source required by the insect larvae.

Chlorosis or yellowing, especially in older needles, is one of the first visible symptoms of *S. noctilio* attack, and is noticeable on pines as soon as two weeks following attack (29). Premature needle senescence and loss is also common, and these responses are readily recapitulated in the laboratory (6,46). Another early and distinctive symptom of attack, especially noticeable in the crowns of impacted trees, is flagging or drooping of young needles at the fascicle sheath (5,6,46). [It should be noted that needle flagging may not be a useful character to assess infection in all pine species as attempts by the authors to reproduce this symptom in a variety other pine species yielded inconsistent results. Field observations of infected red, white and Scots pines in New York also failed to detect noticeable needle flagging (K.J. Dodds, *personal communication*).] Depending on their age, drooping needles may be retained on the tree for some time, sometimes turning red before falling off (29,37). These changes in needle color represent distinct physiological changes in the plant. For example, variations in green color typically indicate damage to the photosynthetic machinery, while red or brown suggests accumulation of flavonoids (65). However, there have not been any studies to define the specific change in needles of *Sirex*-attacked trees at the biochemical or molecular levels. At the

microscopic level, though, non-lignified cells, including phloem and ray parenchyma cells, in *P. radiata* needle segments were shown to collapse and became necrotic within seven days of treatment with *S. noctilio* mucus (66). Such cellular collapse would effectively disrupt transport of carbohydrates and nutrients from the needles to other tissues in the tree.

The effect of *S. noctilio* mucus on needles was replicated in excised pine twigs by several researchers who used it as the basis for bioassays of mucus activity. Thus, genetic and physiological susceptibility of *P. radiata* was gauged by needle flagging and the appearance of chlorosis in detached shoots standing in aqueous solutions of 0.05-0.1 mg/ml mucus (6,67). Such assays were considered more sensitive than tests performed on live trees, and were used to demonstrate that the mucus from *S. noctilio* was more toxic to *P. radiata* than mucus from several other species of woodwasp (68). Respiration, as well as changes in amylase and peroxidase activities, was studied in mucus-treated branch tips (66). These formed the basis of bioassays used to follow activity during biochemical fractionation of the mucus (58).

S. noctilio mucus has a rapid effect on water-relations in attacked pines, causing water tension in needles to drop in a manner similar to girdling (69). Through unknown mechanisms, translocation was inhibited, respiration increased, and both osmotic and turgor pressure were reduced (70). Declines in osmotic pressure were also reported by Iede & Zanetti (71), but increased leaf water pressure (defined as total water potential) was noted in trees showing needle chlorosis (68). Changes in water movement through the transpiration stream following *S. noctilio* attack were assessed by measuring internal tree temperature (72). *P. radiata* trees attacked by *Sirex* had internal temperatures 1-5 °C above air temperature, whereas healthy control trees maintained internal temperatures 2-5 °C below air temperature. This result was attributed to vigorous transpiration in healthy trees in contrast to a “fevered” response due to ineffective

transpiration in attacked trees. Water relations in plants are typically maintained by a delicately balanced process of solute loading and unloading between xylem and phloem tissues (73).

Models have suggested that transpiration is particularly sensitive to reduced sugar loading in photosynthetic tissues at the top of the phloem stream (74).

A general pattern of starch redistribution in which needle starch increases while bark and stem starch decreases has been reported for trees attacked by *S. noctilio* (75). Needles began to accumulate starch within 3-4 days of attack, and by two weeks, starch grains were abundant in the needles of all *P. radiata* trees attacked by *Sirex* (6,46). Treatment with fresh or autoclaved mucus alone reduced the starch in stem samples measured at four weeks (6). Accumulation of starch in needles might fit with reduced sugar loading of the phloem in the model of Holttä *et al.* (74). Respiration in *P. radiata* bark tissues, measured as carbon dioxide release, increased significantly within 2-5 days of attack by caged *S. noctilio* females, and was sustained for at least 16 days following the attack (69). This increased respiration parallels the reported decline in starch content in stem tissues (6). Respiration, measured as an increase in oxygen uptake, was shown to peak 10-15 days following treatment of excised stems with *S. noctilio* mucus (76). The quality of respiration was also reported to change in response to mucus, with an increased ratio of carbon dioxide released to oxygen taken up (66).

Oleoresins are a primary defense against insects and pathogens in conifers (77,78), and total resin flow is the measure most frequently correlated with insect resistance (79). Oleoresin exudation pressure is generally subnormal in trees that have been attacked by *Sirex*, but the observations vary with individual trees (69). Ovipositing *S. noctilio* females frequently drill multiple tunnels, but tunnels containing eggs remain uniformly free of resin, while tunnels that do not contain eggs are often flooded by resin within two or three days (39). Reduced resin

flooding in tunnels containing eggs was ascribed in most cases to the presence of mucus (15,35). However, Coutts & Dolezal (39) mentioned a different substance in egg-containing tunnels that stained intensely with cotton blue [presumably aniline blue used in the lactophenol cotton blue staining technique to detect fungi] and dried with a crazed appearance. Corroborating observations of this other material have not been reported.

Peroxidase and amylase activities were shown to increase in *P. radiata* needles treated with *S. noctilio* mucus (66). Increased amylase activity suggests potential mobilization of stored carbohydrate from starch reserves, while peroxidase induction may reflect activation of defensive responses (80). As previously noted, plant cells respond to the G protein activator, mastoparan (81,82), and the signaling cascades regulated by G proteins control the production of many of the secondary metabolites involved in plant defense responses (83). If a mastoparan-like peptide is present in *S. noctilio* mucus, it could be a primary inductive agent for such defense responses.

Reductions in stem growth rates were reported as a longer term response of pines to *S. noctilio* attack (15,16,18,46,71). Such growth reductions are to be expected considering the degree of damage incurred during oviposition by the vascular cambium, the meristematic tissue responsible for all radial growth in tree stems (84). Radial growth cessation in *P. radiata* attacked by *S. noctilio* is detectable between two and eight weeks post-attack, but the effect is not permanent in resistant trees, and growth may resume within several weeks to several months (15,68). Among seven woodwasps tested, only *S. noctilio* mucus was found to reduce radial growth in *P. radiata* stems (68). Checking of tree growth was reported for *P. patula* attacked by *Sirex* (71), and checking of growth was correlated with an increased attractiveness for *Sirex* woodwasps (14). Tree mortality from severe *Sirex* attacks typically becomes evident within

several months, but trees surviving an unsuccessful attack continue to show measurable decreases in growth relative to trees not attacked (16).

2.4 Tree Responses to *Amylostereum areolatum*

On its own, *Amylostereum areolatum* cannot be considered a particularly aggressive pathogen given the multiple observations of recovery in trees inoculated solely with the fungus (5,23,36). Woodwasp mucus must clearly alter conifer physiology and defense responses in ways that enable the fungus to gain the upper hand and eventually overwhelm the tree. This is not to say, however, that the fungus does not produce specific factors of its own to facilitate the infection. Mycelial growth of *A. areolatum* in pines attacked by *S. noctilio* is preceded by a zone of drying cells (85), but the mechanism for this drying effect is unknown. Drying of the wood tissues appears necessary for the infection as optimal moisture content for growth of the fungus is ~70% saturation -- far lower than the typical conditions inside a tree (15,86). Drying of the tissues precedes mycelial invasion as *A. areolatum* cannot be cultured from either the drying zone or from tissues just beyond it (35,87). Some experimental evidence suggests that *S. noctilio* mucus and the *A. areolatum* fungus are both involved in the drying phenomenon (87). However, drying zones are also observed in conifers infected with the white-rot fungal pathogen, *Heterobasidion annosum*, which is closely related to *A. areolatum* (88,89). *H. annosum* produces several low molecular weight compounds that are toxic to a wide variety of conifers (90,91). Experiments using freshly-cut sections of *P. radiata* stem revealed that ray parenchyma cells were killed in advance of mycelial growth, suggesting that a diffusible agent of fungal origin might be responsible (86). However, it remains to be shown whether toxins of the type produced by *H. annosum* are produced by *A. areolatum*.

Inoculation of *P. taeda* seedlings with *A. areolatum* led to death and collapse of the needles, which subsequently turned gray-green in color and were kinked along the portion of the needle that was still undergoing extension at the time of treatment (92). Gray-green coloration has previously been described for *P. radiata* needles, but in those observations it was unclear whether the color change resulted from the combined effect of the fungus and the mucus (5,29). Intriguingly, needles in non-resistant trees yellowed and senesced quickly, while in resistant trees the younger needles collapsed, turning from green to gray in color (5). This color change in young needles of resistant trees is reminiscent of the hypersensitive response sometimes seen in the leaves of other plants. Evidence for hypersensitivity in pines is beginning to accumulate as major resistance genes for fungal pathogens are identified (93,94). Whether or not a true hypersensitive response mechanism, premised on gene-for-gene interactions, can be found in the *Pinus/Sirex/Amylostereum* pathosystem remains to be seen (95,96).

That said, drying zones suggest that cell death is taking place ahead of the mycelial front, which is to be expected given the necrotrophic lifestyle of *A. areolatum*, but the exact mechanism the fungus uses to cause cell death remains unclear. It may be that fungus stimulates pathways related to the tree's own hypersensitive response system, and then takes advantage of the nutrients released by collapsing cells (97,98). Such a system has been described for at least one other necrotrophic fungus that feeds on a woody species (99). Drying zones might also suggest the involvement of other cellular pathways, such as those that bring about programmed cell death (PCD) (97,100). PCD-type events are presumed to be a normal course of affairs in the development of xylem progenitor cells into water-conductive xylem and wood; however, they must be confirmed by specific tests that distinguish them from other forms of cell death in plants (101).

The importance of polyphenolic compounds in the resistance of pines to *Sirex* attack remains uncertain. *In situ* production of polyphenols at the site of oviposition was described in multiple reports as a pine response to either mechanical wounding or to entry of the fungus (35,76,102,103). However, not all experiments detected differences in polyphenol production between controls and wood infected with *A. areolatum* (104). Polyphenols [visualized using benzidine staining] were produced in the sapwood of infected trees, along the inside edge of the drying zone that precedes advance of the *A. areolatum* mycelia (35). Accumulation of polyphenolic materials was detectable within two weeks of introduction of the fungus, and in resistant trees, accumulation of such materials continued for months after infiltration with the fungus had been stopped (35). Polyphenolic compounds produced by the tree and deposited at the wound or infection site were fungistatic or fungicidal (39,102,105).

Several of the polyphenolic compounds deposited in *Sirex*-attacked wood were purified and identified as stilbenes (14-carbon diphenylethylenes) or phytoalexins (primarily flavonoids) (103). Pinosylvin, a stilbene toxic to *A. areolatum* and many other fungi, was the most abundant polyphenolic compound in the infected zones showing a response (102,104). It may be worth noting that pinosylvin has also been shown to accumulate in pine stems treated with the *H. annosum* toxin, fomannosin (90). The flavonoids, pinobanksin and pinocembrin, were reportedly decreased in the heartwood and knotwood of *Sirex*-attacked trees (102).

There are several intriguing observations in the literature linking enhanced growth of *A. areolatum* to the availability of (undefined) fatty acids from various sources. Titze (106) noted that histological analyses of sectioned bud tissues from the suppressed trees susceptible to *Sirex* attack always showed abundant lipid inclusions in the pith compared to dominant, *Sirex*-resistant trees. Triglycerides, fatty acids and resin acids produced by pines are a nutritional resource

avored by a variety of wood-inhabiting fungi and yeasts, including various *Ophiostoma* (blue-stain) species that are transmitted by bark beetles (107). Because they are good competitors and readily establish themselves on fresh conifer wood chips, where they preferentially metabolize the fatty acids and resins, albino mutants of *Ophiostoma piliferum* are used as biocontrol agents against blue-stain fungi in pulp mill chip piles (108). Surprisingly, there has so far been little effort to follow up on early observations and examine the capacity of *A. areolatum* to metabolize the lipophilic extractives in pine wood. Observations that material (fatty acids?) entering the woodwasp reproductive tract from the Dufour's (oil) gland (57) can stimulate growth of *A. areolatum* in culture remain to be further tested.

2.5 General Responses to Sirex Attack

Plants use a wide variety of mechanisms to deal with pests and disease. Systemic acquired resistance (SAR), induced systemic resistance (ISR), and hypersensitive response (HR) are all possible response paths to which signals from injury or insult may be routed (53). As mentioned previously, *S. noctilio* oviposition induces higher levels of ethylene production in resistant *P. radiata* trees. This suggests that ISR may, in fact, be operative in this pathosystem. However, induced resistance should offer trees enhanced protection from further attacks after an initial encounter with a pest or pathogen, but this has not been the pattern reported for *S. noctilio* attack. While some trees offer far more resistance than others to Sirex, a previously-attacked tree is often weakened, and stress-induced increases in the production of α - and β -pinene increase the chances for subsequent attack (14,38,109).

Ab initio production of polyphenolic compounds occurs at sites where *A. areolatum* is introduced into trees, suggesting that pathogen challenge induces host tree activation of the

phenylpropanoid pathway in part of the defense response (38). Since mechanical wounding alone does not elicit the same results, the host tree must have a recognition system for the fungus. What is unclear is whether this system recognizes factors specific to *A. areolatum* or is just part of a general recognition system for intruding fungi. Fungal elicitors have been shown to induce expression of stilbene synthase genes in Scots pine as part of a general defense response (110). Suspension-cultured cells and seedlings of loblolly and slash pines expressed pathogen-response (PR) genes when exposed to the necrotrophic fungal pathogen *Fusarium subglutinans* f. sp. *pini* (111). These genes also responded to the plant defense signaling molecules, salicylic acid and jasmonic acid. Plant resistance gene homologs have been identified in eastern white pine (*P. strobus*) responding to the blister rust pathogen *Cronartium ribicola* (112). There is no evidence for specific resistance or avirulence genes in host pines or *A. areolatum*. However, dwelling on this would miss the point that without the mucus produced by *S. noctilio*, *A. areolatum* would fail as a pathogen.

2.6 Reducing Tree Susceptibility

Silvicultural practices that promote the health of individual trees (spacing in the canopy, adequate soil conditioning, removing unthrifty individuals) clearly promote resistance to attack by *Sirex noctilio* (14). In particular, the combination of overcrowding and drought leaves trees highly vulnerable to *Sirex* outbreaks (18). Thinning of stands, removal of smaller suppressed trees, and routine monitoring of stands for evidence of woodwasp attack currently comprise the first line of defense to minimize losses to *S. noctilio* (113). Care should also be taken to avoid mechanical injury to trees during thinning and other silvicultural operations in established stands as wounding is a source of attractants for *Sirex* females.

Although evidence in the literature suggests that there might be a genetic basis for resistance to *Sirex noctilio* in pine, conflicting observations make it unclear whether a breeding program could establish robust resistance to Sirex. Many of the pine responses to *A. areolatum* (e.g. increased production of polyphenols, resin, and ethylene), as well as response to environmental factors predisposing trees to attack (e.g. drought tolerance and in-stand competition), are under a degree of genetic control. Some of these responses have been selected for in tree improvement programs looking to enhance resistance to other fungal pathogens. However, the possibilities for breeding resistance to *S. noctilio* mucus have not yet been examined, most likely because the mucus is in limited supply and the specific active factor(s) are unknown. Current techniques in biochemistry, molecular genetics and genomics provide an opportunity unavailable to researchers in the past to probe in this direction. Given the utility of unexpected results from studies of venoms from Aculeata wasp species, it seems almost certain that detailed studies of *Sirex noctilio* will yield unique and useful tools for breeding resistance, as well as probing the physiology and metabolism in cells from conifers and likely other plant species.

Acknowledgements

The authors wish to acknowledge support from the Georgia Traditional Industries Program for Forest Resources and Products (TIP-FRP).

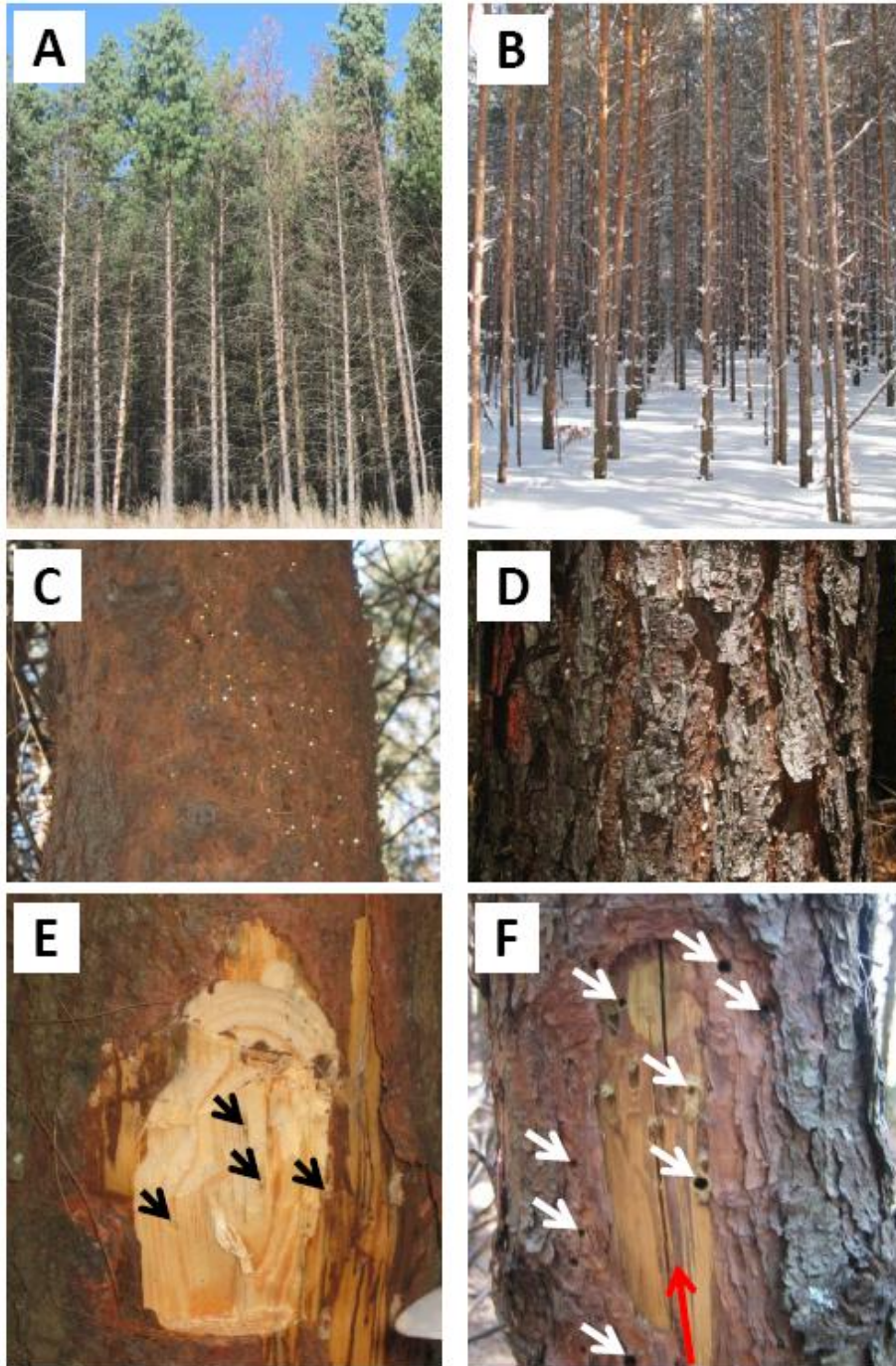


Figure 2.1: *Sirex noctilio* infestations in overstocked pulpwood plantations of *P. patula* in South Africa (panel A) and *P. resinosa* in New York, USA (panel B). Clear resin beads are visible on a live and freshly oviposited *P. resinosa* (panel C), while aged and dried resin remains on dead *P. taeda* in Uruguay (panel D). Galleries and larvae are visible in *P. patula* sapwood once bark and overlay is removed using an axe (panel E, black arrows), while mature wasp exit holes are distinctly visible on dead *P. patula* stems (panel F, white arrows). Note the red patches of discoloration likely due to polyphenol deposition during the defense response (red arrow).

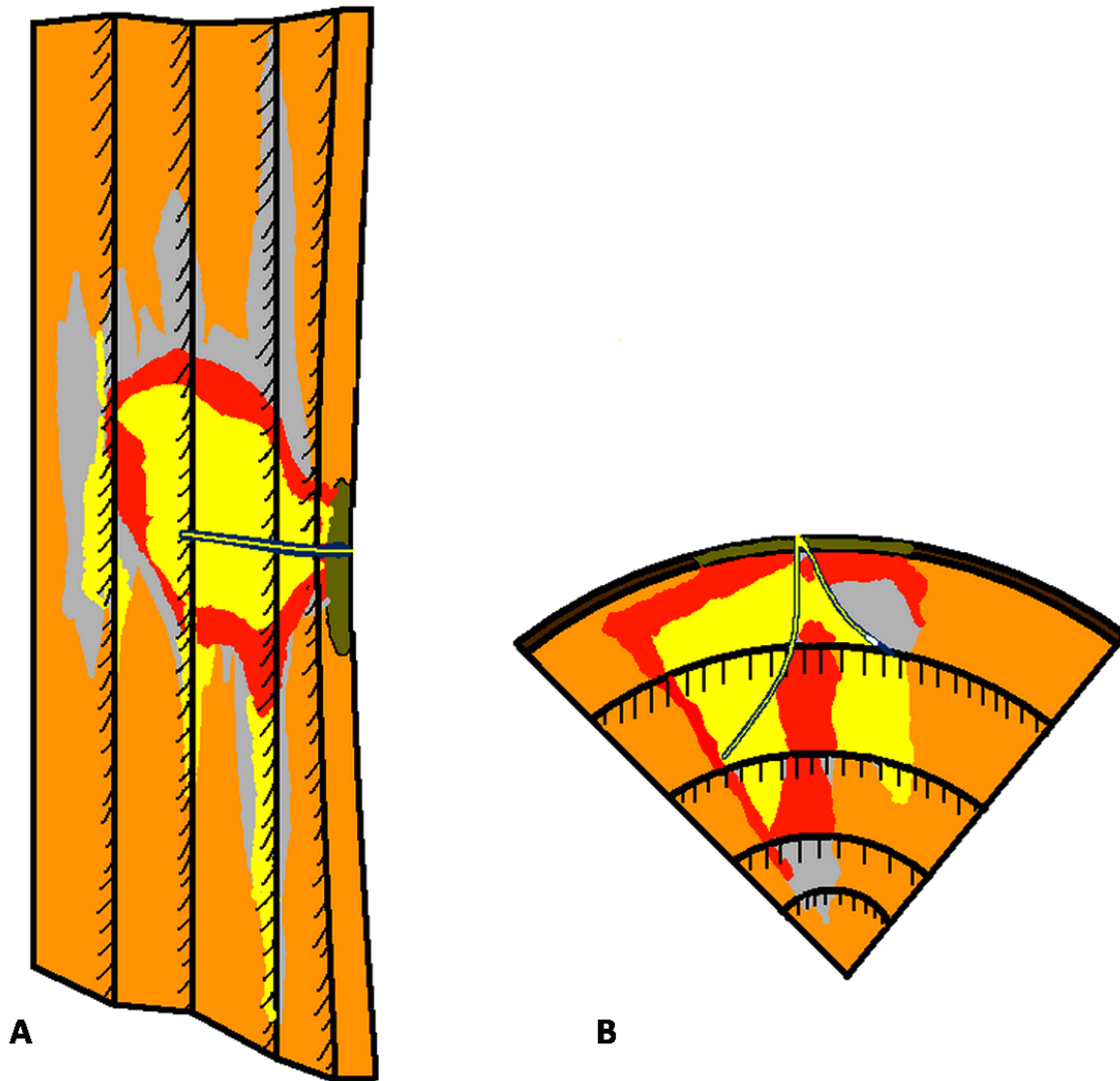


Figure 2.2: Extent of tree responses to *Sirex noctilio* attack. Illustration modeled on figures by Coutts and Dolezal (35). Panel A, radial section of a resistant tree 3 months after woodwasp oviposition. Fungal drying zones (blue-gray), tree-produced polyphenol staining (red), resinosis (yellow), dying cambium and phloem (brown) zones are highlighted with color. Tunnels drilled by the wasp for oviposition are shown in green but should be understood to be resin-flooded. Panel B, transverse section of a resistant tree six weeks after oviposition. Colors are the same as for A. An egg is shown in the shorter of the two tunnels; in a resistant tree, both tunnels would be filled with resin. Oviposition drills are exaggerated in size.

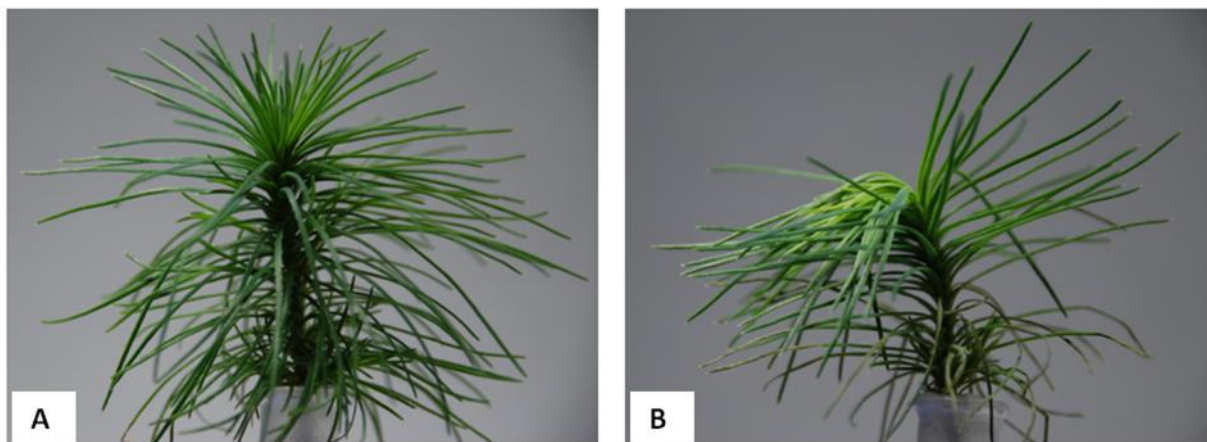


Figure 2.3: Bioassay of *S. noctilio* venom activity using 4-month-old *P. radiata* seedlings, cut at soil line, 7 days after exposure. Panel A, water control. Panel B, aqueous solution of venom.

References

1. Spradbery, J. P., and Kirk, A. A. (1978) Aspects of the Ecology of Siricid Woodwasps (Hymenoptera: Siricidae) in Europe, North Africa and Turkey with Special Reference to the Biological Control of *Sirex noctilio* F. in Australia. *Bulletin of Entomological Research* **68**, 341-359
2. Smith, D. R., and Schiff, N. M. (2002) A review of the siricid woodwasps and their Ibaliid parasitoids (Hymenoptera: Siricidae, Ibaliiidae) in the Eastern United States, with emphasis on the Mid-Atlantic Region (Service, U. F. ed., Southern Research Station
3. Madden, J. L. (1974) Oviposition Behaviour of the Woodwasp, *Sirex noctilio* F. *Australian Journal of Zoology* **22**, 341-351
4. Talbot, P. H. B. (1977) *Sirex-Amylostereum-Pinus* association. *Annual Review of Phytopathology* **15**, 41-54
5. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .1. Effects of symbiotic fungus *Amylostereum* sp. (Thelophoraceae). *Aust. J. Biol. Sci.* **22**, 915-924
6. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .2. Effects of *S. noctilio* mucus. *Aust. J. Biol. Sci.* **22**, 1153-1161
7. Gauld, I. D., Collins, N. M., and Fitton, M. G. (1990) *The Biological Significance and Conservation of Hymenoptera in Europe*, Council of Europe, Strasbourg
8. Borchert, D. F., Glenn; Jackson, Lisa. (2007) Organism Pest Risk Analysis: Risk to the Conterminous United States Associated with the Woodwasp, *Sirex noctilio* Fabricius, and the Symbiotic Fungus, *Amylostereum areolatum* (Fries: Fries) Boidin. USDA-APHIS-PPQ-EDP, Raleigh, NC USA

9. Ciesla, W. M. (2003) European Woodwasp: A Potential Threat to North Americas Conifer Forests. *Journal of Forestry* **101**, 18-23
10. Spradbery, J. P., and Kirk, A. A. (1981) Experimental Studies on the Responses of European Siricid Woodwasps to Host Trees. *Ann. Appl. Biol.* **98**, 179-185
11. APHIS. (2007) Proposed Program for Management of the Woodwasp *Sirex noctilio* Fabricius (Hymenoptera: *Siricidae*), Environmental Assessment. (United States Department of Agriculture, A. a. P. H. I. S. ed., USDA-APHIS, Riverdale, Maryland
12. Dodds, K. J., Cooke, R. R., and Gilmore, D. W. (2007) Silvicultural options to reduce pine susceptibility to attack by a newly detected invasive species, *Sirex noctilio*. *North. J. Appl. For.* **24**, 165-167
13. Rabaglia, R., and Lewis, J. (2006) Economic Analysis of the Potential Impact of *Sirex noctilio*, with Emphasis on Pines in the Southeastern United States: Executive Summary. (Service, F. ed., USDA, Arlington, VA
14. Madden, J. L. (1968) Physiological Aspects of Host Tree Favourability for the Woodwasp, *Sirex noctilio* F. *Proceedings of the Ecological Society of Australia* **3**, 147-149
15. Coutts, M. P., and Dolezal, J. E. (1965) *Sirex noctilio* and the Physiology of *Pinus radiata*. in *38th A.N.Z.A.A.S. Congress*, Hobart, Australia
16. Madden, J. L. (1975) Analysis of an Outbreak of Woodwasp, *Sirex-Noctilio* F (Hymenoptera, Siricidae), in *Pinus-Radiata*. *Bulletin of Entomological Research* **65**, 491-500

17. Coutts, M. P. (1965) *Sirex noctilio* and the physiology of *Pinus radiata*: some studies of the interactions between the insect, the fungus, and the tree in Tasmania. in *Forestry and Timber Bureau* (D.N.D. ed., Forest Research Institute, Canberra, Australia
18. Gilmour, J. W. (1965) The life cycle of the fungal symbiont of *Sirex noctilio*. *New Zealand Journal of Forestry* **10**, 80-89
19. Wermelinger, B., Rigling, A., Schneider-Mathis, D., and Dobbertin, M. (2008) Assessing the Role of Bark- and Wood-Boring Insects in the Decline of Scots Pine (*Pinus sylvestris*) in the Swiss Rhone Valley. *Ecological Entomology* **33**, 239-249
20. Dicke, M., and Hilker, M. (2003) Induced plant defences: from molecular biology to evolutionary ecology. *Basic Appl. Ecol.* **4**, 3-14
21. Howe, G. A. (2004) Jasmonates as signals in the wound response. *J. Plant Growth Reg.* **23**, 223-237
22. Dodds, K. J. (2006) *Sirex noctilio* trapping protocols. U. S. Forest Service, Durham, NH
23. Madden, J. L. (1971) Some treatments which render Monterey pine (*Pinus radiata*) attractive to the woodwasp *Sirex noctilio*. *Bulletin of Entomological Research* **60**, 467-472
24. Morgan, F. D., and Stewart, N. C. (1972) Developing and testing a lure-trap for the woodwasp *Sirex noctilio* F. *Australian Forestry* **36**, 38-46
25. Simpson, R., and McQuilkin, R. (1976) Identification of volatiles from felled *Pinus radiata* and the electroantennograms they elicit from *Sirex noctilio*. *Entomologia Experimentalis et Applicata* **19**, 205-213

26. Simpson, R. (1976) Bioassay of pine oil components as attractants for *Sirex noctilio* (Hymenoptera: Siricidae) using electroantennogram techniques. *Entomologia Experimentalis et Applicata* **19**, 11-18
27. Neumann, F. G., Harris, J. A., Kassaby, F. Y., and Minko, G. (1982) An improved technique for early detection and control of the Sirex wood wasp in radiata pine plantations. *Australian Forestry* **45**, 117-124
28. Morgan, F. D. (1968) Bionomics of Siricidae. *Annu. Rev. Entomol.* **13**, 239-256
29. Rawlings, G. B. (1948) Recent Observations on the *Sirex noctilio* Population in *Pinus radiata* Forests in New Zealand. *New Zealand Journal of Forestry* **5**, 411-421
30. Vaartaja, O., and King, J. (1964) Inoculation Experiments with *Amylostereum* sp. from a Woodwasp. *Plant Disease Reporter* **48**, 438-440
31. Coutts, M. P., and Dolezal, J. E. (1965) *Sirex noctilio*, its associated fungus, and some aspects of wood moisture content. *Aust. For. Res.* **1**, 3-13
32. Dunn, J. P., and Lorio, P. L. (1993) Modified water regimes affect photosynthesis, xylem water potential, cambial growth, and resistance of juvenile *Pinus taeda* L to *Dendroctonus frontalis* (Coleoptera, Scolytidae). *Environmental Entomology* **22**, 948-957
33. Lombardero, M. J., Ayres, M. P., Lorio, P. L., and Ruel, J. J. (2000) Environmental effects on constitutive and inducible resin defences of *Pinus taeda*. *Ecology Letters* **3**
34. Turtola, S., Manninen, A. M., Rikala, R., and Kainulainen, P. (2003) Drought stress alters the concentration of wood terpenoids in Scots pine and Norway spruce seedlings. *J. Chem. Ecol.* **29**, 1981-1995

35. Coutts, M. P. D., J. E. (1966) Polyphenols and resin in the resistance mechanism of *Pinus radiata* attacked by the wood wasp, *Sirex noctilio*, and its associated fungus. (Department of National Development, F. a. T. B. ed., Commonwealth of Australia)
36. Titze, J. F., and Stahl, W. (1970) Inoculations of *Pinus radiata* trees with *Amylostereum areolatum*, the symbiotic fungus of *Sirex noctilio*. *Aust. For. Res.* **4**, 3-14
37. Coutts, M. P. (1965) *Sirex noctilio* and the Physiology of *Pinus radiata*: Some Studies of the Interactions Between the Insect, the Fungus, and the Tree in Tasmania. (Bureau, N. D. F. a. T. ed., Forest Research Institute, Canberra, Australia)
38. Madden, J. L. (1988) *Sirex* in Australasia. in *Dynamics of Forest Insect Populations* (Berryman, A. A. ed.), Plenum Publishing Corporation, New York. pp 407-429
39. Coutts, M. P., and Dolezal, J. E. (1969) Emplacement of fungal spores by the woodwasp, *Sirex noctilio*, during oviposition. *Forest Science* **15**, 412-&
40. Zondag, R., and Nuttall, M. J. (1977) *Sirex noctilio* Fabricius (Hymenoptera:Siricidae). (Service, N. Z. F. ed., Forest Research Institute)
41. Michelozzi, M. (1999) Defensive roles of terpenoid mixtures in conifers. *Acta Botanica Gallica* **146**, 73-84
42. DeBruxelles, G. L., and Roberts, M. R. (2001) Signals regulating multiple responses to wounding and herbivores. *Critical Reviews in Plant Sciences* **20**, 487-521
43. Maffei, M. E., Mithofer, A., and Boland, W. (2007) Insects feeding on plants: Rapid signals and responses preceding the induction of phytochemical release. *Phytochemistry* **68**, 2946-2959
44. Wasternack, C. (2007) Jasmonates: An update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annals of Botany* **100**, 681-697

45. Eldridge, R. H., and Taylor, E. E. (1989) *Sirex woodwasp - a pest of pine in NSW*. (Commission, F. ed., Forestry Commission of New South Wales, New South Wales)
46. Coutts, M. P. (1968) Rapid physiological change in *Pinus radiata* following attack by *Sirex noctilio* and its associated fungus, *Amylostereum* sp. *Aust. J. Sci.* **30**, 275-277
47. Kopke, D., Schroder, R., Fischer, H. M., Gershenzon, J., Hilker, M., and Schmidt, A. (2008) Does egg deposition by herbivorous pine sawflies affect transcription of sesquiterpene synthases in pine? *Planta* **228**, 427-438
48. Roberds, J. H., Strom, B. L., Hain, F. P., Gwaze, D. P., McKeand, S. E., and Lott, L. H. (2003) Estimates of genetic parameters for oleoresin and growth traits in juvenile loblolly pine. *Can. J. For. Res.* **33**, 2469-2476
49. Rosner, S., and Hannrup, B. (2004) Resin canal traits relevant for constitutive resistance of Norway spruce against bark beetles: environmental and genetic variability. *For. Ecol. Manage.* **200**, 77-87
50. Heijari, J., Nerg, A. M., Kainulainen, P., Vuorinen, M., and Holopainen, J. K. (2008) Long-term effects of exogenous methyl jasmonate application on Scots pine (*Pinus sylvestris*) needle chemical defence and diprionid sawfly performance. *Entomologia Experimentalis et Applicata* **128**, 162-171
51. Harju, A. M., Venalainen, M., Laakso, T., and Saranpaa, P. (2009) Wounding response in xylem of Scots pine seedlings shows wide genetic variation and connection with the constitutive defence of heartwood. *Tree Physiol.* **29**, 19-25
52. Ecker, J. R., and Davis, R. W. (1987) Plant Defense Genes Are Regulated by Ethylene. *Proc. Natl. Acad. Sci. USA* **84**, 5202-5206

53. Walters, D., and Heil, M. (2007) Costs and Trade-offs Associated with Induced Resistance. *Physiol. Mol. Plant Pathol.* **71**, 3-17
54. Shain, L., and Hillis, W. E. (1972) Ethylene Production in *Pinus-Radiata* in Response to *Sirex-Amylostereum* Attack. *Phytopathology* **62**, 1407-1409
55. Nagy, N. E., Krokene, P., and Solheim, H. (2006) Anatomical-based defense responses of Scots pine (*Pinus sylvestris*) stems to two fungal pathogens. *Tree Physiol.* **26**, 159-167
56. Phillips, M. A., Bohlmann, J., and Gershenzon, J. (2006) Molecular regulation of induced terpenoid biosynthesis in conifers. *Phytochemistry Reviews* **5**, 179-189
57. Boros, C. B. (1968) *The relationship between the woodwasp Sirex noctilio F. and the wood-rot fungus Amylostereum sp.* Master of Agricultural Science M. S., University of Adelaide
58. Wong, L. K., and Crowden, R. K. (1976) Preliminary studies on mucus secretion of wood wasp, *Sirex noctilio* F .1. Physicochemical and biochemical properties. *Aust. J. Biol. Sci.* **29**, 21-32
59. Pimenta, A. M. C., and DeLima, M. E. (2005) Small peptides, big world: biotechnological potential in neglected bioactive peptides from arthropod venoms. *Journal of Peptide Science* **11**, 670-676
60. Tomita, U., Inanobe, A., Kobayashi, I., Takahashi, K., Ui, M., and Katada, T. (1991) Direct interactions of mastoparan and compound 48/80 with GTP-binding proteins. *Journal of Biochemistry (Tokyo)* **109**, 184-189
61. Miles, G. P., Samuel, M. A., Jones, A. M., and Ellis, B. (2004) Mastoparan rapidly activates plant MAP kinase signaling independent of heterotrimeric G proteins. *Plant Physiology and Biochemistry* **134**, 1332-1336

62. Guo, Y. F., and Gan, S. S. (2005) Leaf senescence: signals, execution, and regulation. *Current Topics in Developmental Biology* **71**, 83-112
63. Kuhn-Nentwig, L. (2003) Antimicrobial and cytolytic peptides of venomous arthropods. *Cell. Mol. Life Sci.* **60**, 2651-2668
64. Kellner, R. L. L. (2002) The role of microorganisms for eggs and progeny. in *Chemoecology of Insect Eggs and Egg Deposition* (Hilker, M., and Meiners, T. eds.), Blackwell Publishing Ltd., Oxford, UK. pp 149-167
65. Gunthardt-Goerg, M. S., and Vollenweider, P. (2007) Linking Stress with Macroscopic and Microscopic Leaf Response in Trees: New Diagnostic Perspectives. Elsevier Sci Ltd
66. Fong, L. K., and Crowden, R. K. (1973) Physiological effects of mucus from wood wasp, *Sirex noctilio* F., on foliage of *Pinus radiata* D. Don. *Aust. J. Biol. Sci.* **26**, 365-378
67. Bowling, J. P., and Dolezal, J. E. (1970) Initial Note on the Development of a "Mucus Test" to Determine Resistance of *Pinus radiata* to Attack by *Sirex noctilio*. *Australian Forestry Research* **5**, 57-62
68. Spradbery, J. P. (1973) A comparative study of the phytotoxic effects of siricid woodwasps on conifers. *Ann. Appl. Biol.* **75**, 309-320
69. Madden, J. L. (1977) Physiological Reactions of *Pinus radiata* to Attack by Woodwasp, *Sirex noctilio* F. (Hymenoptera-Siricidae). *Bulletin of Entomological Research* **67**, 405-426
70. Madden, J. L., Coutts, M.P. (1979) The Role of Fungi in the Biology and Ecology of Woodwasps (Hymenoptera: Siricidae). in *Insect-fungus symbiosis : nutrition, mutualism, and commensalism : proceedings of a symposium* (Batra, L. R. ed.), Halsted Press, Montclair, NJ. pp 165-174

71. Iede, E. T., and Zanetti, R. (2007) Occurrence and management recommendations of *Sirex noctilio* Fabricius (Hymenoptera, Siricidae) on *Pinus patula* (Pinaceae) plantations in the state of Minas Gerais, Brazil. *Rev. Bras. Entomol.* **51**, 529-531
72. Jamieson, J. P. S. (1957) Some Observations on the Relation of the Internal Temperature of *P.radiata* to Infestation by *Sirex*. *New Zealand Journal of Forestry* **VII**, 32-34
73. DeBoer, A. H., and Volkov, V. (2003) Logistics of water and salt transport through the plant: structure and functioning of the xylem. *Plant Cell & Environment* **26**, 87-101
74. Holttä, T., Vesala, T., Sevanto, S., Peramak, M., and Nikinmaa, E. (2006) Modeling xylem and phloem water flows in trees according to cohesion theory and Munch hypothesis. *Trees - Structure & Function* **20**, 67-78
75. Gilbertson, R. L. (1984) Relationships between insects and wood-rotting basidiomycetes. in *Fungus-insect relationships : perspectives in ecology and evolution* (Wheeler, Q., and Blackwell, M. eds.), Columbia University Press, New York. pp 130-165
76. Coutts, M. P. (1970) The physiological effects of the mucus secretion of *Sirex noctilio* on *Pinus radiata*. *Aust. For. Res.* **4**, 23-26
77. Gijzen, M., Lewinsohn, E., Savage, T. J., and Croteau, R. B. (1993) Conifer monoterpenes - biochemistry and bark beetle chemical ecology. in *Bioactive Volatile Compounds from Plants*
78. Keeling, C. I., and Bohlmann, J. (2006) Diterpene resin acids in conifers. *Phytochemistry Reviews* **67**, 2415-2423
79. Tisdale, R. A., Nebeker, T. E., and Hodges, J. D. (2003) The role of oleoresin flow in the induced response of loblolly pine to a southern pine beetle associated fungus. *Canadian Journal of Botany-Revue Canadienne De Botanique* **81**, 368-374

80. Kawano, T. (2003) Roles of the Reactive Oxygen Species-Generating Peroxidase Reactions in Plant Defense and Growth Induction. *Plant Cell Reports* **21**, 829-837
81. Cho, M. H., Tan, Z., Erneux, C., Shears, S. B., and Boss, W. F. (1995) The effects of mastoparan on the carrot cell plasma membrane polyphosphoinositide phospholipase-C. *Plant Physiol.* **107**, 845-856
82. Legendre, L., Yueh, Y. G., Crain, R., Haddock, N., Heinstein, P. F., and Low, P. S. (1993) Phospholipase-C Activation During Elicitation of the Oxidative Burst in Plant Cells. *J. Biol. Chem.* **268**, 24559-24563
83. Zhao, J., Davis, L. C., and Verpoorte, R. (2005) Elicitor Signal Transduction Leading to Production of Plant Secondary Metabolites. *Biotechnology Advances* **23**, 283-333
84. Barbosa, P., and Wagner, M. R. (1989) *Introduction to Forest and Shade Tree Insects*, Academic Press, San Diego
85. Coutts, M. P., and Dolezal, J. E. (1965) *Sirex noctilio*, its Associated Fungus, and Some Aspects of Wood Moisture Content. *Australian Forestry Research* **1**, 3-13
86. King, J. M. (1966) Some Aspects of Biology of Fungal Symbiont of *Sirex Noctilio*. *Australian Journal of Botany* **14**, 25-&
87. Kile, G. A., and Turnbull, C. R. A. (1974) Drying in the sapwood of radiata pine after inoculation with *Amylostereum areolatum* and *Sirex mucus*. *Aust. For. Res.* **6**, 35-40
88. Coutts, M. P. (1976) Formation of Dry Zones in Sapwood of Conifers .1. Induction of Drying in Standing Trees and Logs by *Fomes Annosus* and Extracts of Infected Wood. *European Journal of Forest Pathology* **6**, 372-381
89. Majjala, P., Harrington, T. C., and Raudaskoski, M. (2003) A Peroxidase Gene Family and Gene Trees in *Heterobasidion* and Related Genera. *Mycologia* **95**, 209-221

90. Bassett, C., Sherwood, R. T., Kepler, J. A., and Hamilton, P. B. (1967) Production and biological activity of fomannosin, a toxic sesquiterpene metabolite of *Fomes annosus*. *Phytopathology* **57**
91. Sonnenbichler, J., Bliestle, I. M., Peipp, H., and Holdenrieder, O. (1989) Secondary fungal metabolites and their biological activities I. Isolation of antibiotic compounds from cultures of *Heterobasidion annosum* synthesized in the presence of antagonistic fungi or host plant cells. *Biological Chemistry Hoppe-Seyler* **370**, 1295-1303
92. Bordeaux, J. M. (2008) *Characterization of Growth Conditions for Production of a Laccase-Like Phenoloxidase by Amylostereum areolatum, a Fungal Pathogen of Pines and Other Conifers*. Master of Science, University of Georgia
93. Kubisiak, T. L., HV, A., and CD, N. (2005) Genetic interaction of the fusiform rust fungus with resistance gene Fr1 in loblolly pine. *Phytopathology* **95**, 376-380
94. Liu, J. J., and Ekramoddoullah, A. K. M. (2004) Isolation, genetic variation and expression of TIR-NBS-LRR resistance gene analogs from western white pine (*Pinus monticola* Dougl. ex. D. Don.). *Molecular Genetics and Genomics* **270**, 432-441
95. Mur, L. A. J., Kenton, P., Lloyd, A. J., Ougham, H., and Prats, E. (2008) *The Hypersensitive Response: the Centenary is Upon Us but How Much Do We Know?* , Oxford Univ Press
96. Hammond-Kosack, K. E., and Jones, J. D. G. (1997) Plant Disease Resistance Genes. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 575-607
97. Levine, A., Pennell, R. I., Alvarez, M. E., Palmer, R., and Lamb, C. (1996) Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. *Current Biology* **6**, 427-437

98. Mayer, A. M., Staples, R. C., and Gil-ad, N. L. (2001) Mechanisms of survival of necrotrophic fungal plant pathogens in hosts expressing the hypersensitive response. *Phytochemistry* **58**, 33-41
99. Govrin, E. M., and Levine, A. (2000) The Hypersensitive Response Facilitates Plant Infection by the Necrotrophic Pathogen *Botrytis cinerea*. *Current Biology* **10**, 751-757
100. Khurana, S. M. P., Pandey, S. K., Sarkar, D., and Chanemougasoundharam, A. (2005) Apoptosis in Plant Disease Response: A Close Encounter of the Pathogen Kind. *Curr. Sci.* **88**, 740-752
101. Reape, T. J., and McCabe, P. F. (2008) Apoptotic-Like Programmed Cell Death in Plants. *New Phytologist* **180**, 13-26
102. Hillis, W. E., and Inoue, T. (1968) Formation of Polyphenols in Trees .4. Polyphenols Formed in *Pinus radiata* after Sirex Attack. *Phytochemistry* **7**, 13-&
103. Hart, J. H. (1981) Role of Phytostilbenes in Decay and Disease Resistance. *Annual Review of Phytopathology* **19**, 437-458
104. Coutts, M. P. (1969) The formation of polyphenols in small blocks of *Pinus radiata* sapwood with and without the fungal symbiont of *Sirex*. *Aust. For. Res.* **4**, 29-34
105. Himejima, M., Hobson, K. R., Otsuka, T., Wood, D. L., and Kubo, I. (1992) Antimicrobial terpenes from oleoresin of ponderosa pine tree *Pinus ponderosa* - a defense-mechanism against microbial invasion. *J. Chem. Ecol.* **18**, 1809-1818
106. Titze, J. F. (1965) Physiological suppression in *Pinus radiata* and its susceptibility to *Sirex*. *Aust. For. Res.* **1**, 51-55

107. Gao, Y., Breuil, C., and Chen, T. (1994) Utilization of triglycerides, fatty-acids and resin acids in lodgepole pine wood by a sapstaining fungus *Ophiostoma piceae*. *Mater. Organismen* **28**, 105-118
108. Blanchette, R. A., Farrell, R. L., Burnes, T. A., Wendler, P. A., Zimmerman, W., Brush, T. S., and Snyder, R. A. (1992) Biological control of pitch in pulp and paper-production by *Ophiostoma piliferum*. *TAPPI J* **75**, 102-106
109. Coutts, M. P., and Dolezal, J. E. (1966) Some Effects of Bark Cincturing on the Physiology of *Pinus radiata*, and on *Sirex* Attack. *Aust. For. Res.* **2**, 17-28
110. Preisig-Mueller, R., Axel, S., Ilka, B., Hans-Jörg, R., and Helmut, K. (1999) Characterization of a pine multigene family containing elicitor- responsive stilbene synthase genes. *Plant Molecular Biology* **V39**, 221-229
111. Davis, J. M., Wu, H., Cooke, J. E. K., Reed, J. M., Luce, K. S., and Michler, C. H. (2002) Pathogen Challenge, Salicylic Acid, and Jasmonic Acid Regulate Expression of Chitinase Gene Homologs in Pine. *Molecular Plant-Microbe Interactions* **15**, 380-387
112. Smith, J. A., Blanchette, R. A., Burnes, T. A., Jacobs, J. J., Higgins, L., Witthuhn, B. A., David, A. J., and Gillman, J. H. (2006) Proteomic comparison of needles from blister rust-resistant and susceptible *Pinus strobus* seedlings reveals UpRegulation of putative disease resistance proteins. *Molecular Plant-Microbe Interactions* **19**, 150-160
113. Dodds, K. I., Cooke, R. R., and Gilmore, D. W. (2007) Silvicultural Options to Reduce Pine Susceptibility to Attack by a Newly Detected Invasive Species, *Sirex noctilio*. *North. J. Appl. For.* **24**, 165-167

CHAPTER 3

BIOMARKER GENES HIGHLIGHT INTRASPECIFIC AND INTERSPECIFIC VARIATIONS IN THE RESPONSES OF PINUS TAEDA L. AND PINUS RADIATA D. DON TO SIREX NOCTILIO F. ACID GLAND SECRETIONS²

² Bordeaux, J.M., W.W. Lorenz and J.F.D. Dean, 2012, Tree Physiology, 32 (10), 1302-1312.
Reprinted here with permission of publisher.

Abstract

Sirex noctilio F., a Eurasian horntail woodwasp recently introduced into North America, oviposits in pines and other conifers and in the process spreads a phytopathogenic fungus that serves as a food source for its larvae. During oviposition the woodwasp also deposits a mucus produced in its acid (venom) gland that alters pine defense responses and facilitates infection by the fungus. A 26,496-feature loblolly pine cDNA microarray was used to survey gene expression of pine tissue responding to *S. noctilio* venom. Six genes were selected for further assessment by qRT-PCR, including one that encoded an apparent PR-4 protein and another that encoded a thaumatin-like protein. Expression of both was strongly induced in response to venom, while expression of an apparent actin gene (ACT1) was stable in response to the venom. The pattern of gene response was similar in *Pinus taeda* L. and *P. radiata* D. Don, but the magnitude of response in *P. radiata* was significantly stronger for each of the induced genes. The magnitude of biomarker gene response to venom also varied according to genotype within these two species. The qRT-PCR assay was used to demonstrate that the primary bioactive component in *S. noctilio* venom is a polypeptide.

3.1 Introduction

The Eurasian horntail woodwasp, *Sirex noctilio* Fabricius (Hymenoptera: Siricidae), an invasive insect recently introduced into North America, represents a serious economic and ecological threat to pine forests throughout the U.S. and Canada ((1-4). Females of the species typically attack dead or dying pines in their native range (Europe, Asia, and northern Africa), where they cause little if any economic impact (5,6). The relative importance of *Sirex noctilio* to forest health in Europe may be measured by the fact that it is sometimes neglected entirely in discussions of European forest pests (7). By contrast, *S. noctilio* is a major pest when introduced as an exotic into commercial pine plantations of the southern hemisphere (8). These southern hemisphere pine plantations are typically stocked with North American pine species, mostly Monterey (*P. radiata* D. Don) and loblolly pines (*P. taeda* L.), which highlights the danger this woodwasp may pose for North American forests (7).

Unlike other members of the Siricidae family, *S. noctilio* attacks conifers exclusively (8). *S. noctilio* attack occurs via the act of oviposition in which the female woodwasps use their specialized ovipositors to drill tunnels into the xylem and deposit their eggs and venom along with arthrospores (oidia) of a fungal symbiont, the white-rot basidiomycete, *Amylostereum areolatum* (6,9,10). While both the venom and the fungus must be present to kill trees (9,11-13), the venom alone causes overt changes in host tree phenotype, such as the needle droop (flagging) characteristic of *S. noctilio* attack, as well as physiological changes that serve to depress host defense responses (10,11a,b). Since the apparent function of *S. noctilio* venom is to weaken the tree sufficiently that the fungus can overcome any remaining defense response (10), an understanding of the venom mode of action will be important for unraveling the mechanism of pathology and possibly developing new measures to intercede in this pathosystem.

Bioassays for the effect of *S. noctilio* venom on pines have typically relied on the visual observation of responses, such as needle flagging and chlorosis (14). A variety of physiological measurements, including needle starch accumulation, dry-weight change, respiration rate, moisture content, amylase and peroxidase activity, leaf pressure (total water potential), and radial stem growth have also been used in the past to follow the effects of *S. noctilio* venom on treated pines (12,13,15). Venom action has been tested using intact trees (10,12,13,15), but such assays are impractical for routine use. Branch and needle explants from various species of pine have been used as indicators of venom activity with varying sensitivity (10,12,15-18), but there exist few reliable quantitative measures for testing *S. noctilio* venom activity. An issue complicating development of consistent, reproducible bioassays has been tree-to-tree variability in response to venom. Although discussed in little detail, reports in the older *Sirex* literature noted that some *P. radiata* trees were more sensitive than others to the application of *S. noctilio* venom (9,10,17). Noting this variation, researchers used explants from susceptible trees in subsequent studies (12,15,16,18). It remains unclear whether these observed variations in venom sensitivity were linked to genotype or some other factor.

The importance of *S. noctilio* venom to this pathosystem is reflected in the oversized venom reservoir that fills a large proportion of the abdomen in newly emergent female woodwasps. Despite the absence of a reliable quantitative assay for bioactivity, early biochemical studies characterized the venom as a protein-polysaccharide complex presenting several enzyme activities, including amylase, esterase, phenoloxidase, and protease (16). Chemical tests were used to show that non-sulfated acidic mucopolysaccharide is a primary constituent of *S. noctilio* venom. Activity tests showed the active factor in venom to be heat-stable, retaining activity after boiling for at least two hours and even after autoclaving (10,13). In

fact, heating appeared to enhance the activity of diluted venom, which may be related to the slow disaggregation described for the active factor into a low molecular mass fraction (2-5 kDa) after dilution in water (15,16). This disaggregated factor is most likely the venom component that is transported from the site of woodwasp oviposition on pine stems to photosynthetic tissues in the upper branches of the tree. Venoms of the more familiar aculeate (stinging) wasps typically are complex, containing large and small polypeptides, biogenic amines (histamine, dopamine, adrenaline, noradrenaline, and serotonin), as well as enzymes such as phospholipases and hyaluronidases (19). However, the biochemical nature of the active factor in *S. noctilio* venom has remained, until now, unclear.

To better understand *S. noctilio* venom and its mode of action in *Pinus* species, work was undertaken to develop biomarker assays with which to follow venom activity. These biomarker assays were used to investigate the variability in venom responses between individual trees and in different pine species. Additional studies tested whether or not polypeptide components in *S. noctilio* venom are important for venom effects on pines.

3.2 Materials and methods

3.2.1 Biological materials

Loblolly pine (*Pinus taeda*) or Monterey pine (*Pinus radiata*) seeds were soaked in a 1% (v:v) hydrogen peroxide solution at 4° C for four days to hasten germination (20). Open-pollinated (OP) *P. taeda* seeds from a 7-56 genotype mother tree were kindly provided by Dr. Michael Cunningham (International Paper Co., Bainbridge, GA), while OP *P. radiata* seeds were provided by Dr. Kathleen Jermstad (U.S. Forest Service, Placerville, CA). Seeds were planted in commercial potting soil, and at six weeks the seedlings, having developed substantial tap roots,

were transferred to Ray Leach Conetainers (Stuewe & Sons, Tangent, OR). Seedlings were thereafter kept in growth chambers under a 16 h light/8 h diurnal cycle and watered every three to four days. Fertilizer was applied every four weeks as a solution of 14.5 g Miracle-Gro (Scotts, Marysville, OH) and 1.2 g chelated iron per 4 l water. For the *P. radiata* genotypic variation study, 17 individuals were kept in a greenhouse under ambient conditions and hedged at regular intervals to produce multiple shoot tips. Phenotype assays were performed using individual shoot tips with cut ends submerged in 50 μ l of 20 mg/ml *S. noctilio* venom and then transferred to water after venom absorption. To minimize false positives during the incubation period, cut ends were regularly trimmed at the bottom and water was refreshed frequently to prevent tracheal element clogging by microbial growth. The 17 *P. radiata* individuals were designated as either venom-sensitive or non-responsive based on phenotype (observable wilting and color change in needles). Two venom-sensitive trees and one non-responsive tree were selected for routine use in the PCR biomarker assays.

S. noctilio females provided by Kelley Zylstra (USDA APHIS, Syracuse, NY) were frozen immediately upon emergence from pine logs and stored at -80° C until use. Frozen wasps were dipped in ethanol prior to dissection to remove the venom sac and glands. Pooled venom tissues were ground in water using a fritted-glass homogenizing tube and brought to a concentration of 20 mg/ml whole tissue in deionized water. The resultant solution was boiled for 15 min, cooled and dispensed into 1-ml aliquots prior to storage at -20° C until use. Prior to performing assays, the venom solution was thawed and then centrifuged at 17,000 g for 5-10 min to remove any precipitate. Only the clarified supernatant was used in subsequent assays.

3.2.2 Pine cDNA microarray analysis

To generate a short list of candidate biomarker genes in loblolly pine that respond strongly to *S. noctilio* venom exposure a minimal microarray experiment was performed. The experiment used the PtGen2 loblolly pine microarray platform, which contains 26,496 features representing 25,848 unique cDNA amplimers and a unigene set of about 16,000 genes (Lorenz et al. 2011) and employed a reference design (21) to identify genes differentially expressed between venom-treated and control seedlings at two different time points. The reference sample was comprised of total RNA isolated from *P. taeda* roots, shoots, and needles (Lorenz et al. 2011). As the sole purpose of this experiment was to generate a list of potential biomarkers for PCR assays, the experimental design comprised two biological replicates of control and time point samples, as well as two technical replicates. The data from this microarray experiment are not of such statistical significance as to be useful for genome-scale analyses on their own, and their utility is unpredictable beyond the narrow scope of our need to identify useful biomarkers; nonetheless, the data has been placed in the GEO database at NCBI under accession number_GSE40000.

To generate biological samples for the microarrays, sixty 5-month-old *P. taeda* seedlings were cut at the soil line and cut ends were placed in 50 µl each of 20 mg/ml *S. noctilio* venom or water. After uptake, seedlings were transferred to water until samples were collected. At two time points after treatment (24, 48 h), two sets of 10 venom-treated seedlings were pooled, as was one set of 10 water control seedlings. This yielded a total of six samples for microarray analysis. The seedling pools from each time point were collected and flash-frozen in liquid nitrogen. Frozen tissue was ground using a SPEX model 6850 freezer mill (SPEX, Metuchen, NJ), and RNA was extracted using a protocol modified from Chang et al. (22,23). Briefly, 3 g of

ground tissue was transferred to 20 ml RNA extraction buffer (2 % CTAB (w:v), 2 % (w:v) polyvinyl pyrrolidone, 100 mM Tris-HCL pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 g/l spermidine, and 2 % (v:v) β -mercaptoethanol), extracted twice with chloroform and centrifuged, with retention of the aqueous layer after each centrifugation step. RNA was precipitated from the aqueous pool by incubation overnight with 10 M LiCl, centrifuged and the pellet was resuspended in SSTE buffer. Resuspended RNA was further purified by phenol/chloroform, pH 8 (PC8) and neat chloroform extraction, centrifugation and retention of the aqueous layer. As a final step, RNA was precipitated in ethanol, resuspended in water, and treated with RNase-free DNase (TurboDNA Free, Ambion, Austin, TX). Complementary DNA was synthesized with incorporation of amino-allyl and amino-hexyl modified nucleotides using Invitrogen's SuperscriptTM Indirect Labeling Kit (Invitrogen, Carlsbad, CA). Cy-5 and Cy-3 dyes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) were suspended in 80 μ l DMSO and coupled to the modified cDNA per manufacturer's instructions. Dye-coupled cDNAs were purified using published methods (24) with mass and labeling efficiencies quantified spectrophotometrically. Microarrays were hybridized with labeled targets as described (25). Slides were incubated in a HybChamberTM (Genomic Solutions, Ann Arbor, MI) containing 20 μ l 100 mM dithiothreitol. HybChambers were wrapped in foil and incubated in a 48 °C shaking water bath at 50 rpm for 14-16 h.

A ProScanArrayTM confocal scanner (Perkin Elmer, Waltham, MA) was used to scan microarrays at 532 nm and 635 nm. ImaGene software (ver. 7.5, BioDiscovery Inc., El Segundo, CA, USA) was used to process raw fluorescence data. Data were filtered, log-transformed, normalized (print tip-Lowess) and statistical analyses were performed using BRB-Array Tools Ver. 8.0 (26). Spot signals were filtered and flagged as described previously (24,27), and probes

with $\geq 50\%$ missing data were eliminated from further analyses. Differentially expressed genes were identified by comparing two treatment groups (venom treatment vs. water) with a test of random variance (26) using a false discovery rate (FDR) of 0.5 and a differential expression cut-off minimum of 1.5-fold.

3.2.3 Biomarker gene analysis

Microarray analysis identified genes whose expression was up-regulated in pine seedlings exposed to *S. noctilio* venom. From a list of the most highly up-regulated genes (Table 3.1), three targets were chosen for further testing as potential biomarkers. Two of the genes were annotated as a putative PR-4 (PR4) protein and a thaumatin-like protein (TLP), while one potential biomarker was not annotated (unknown, UNK). Two additional genes annotated as actin1 (ACT1) and actin2 (ACT2), were chosen for potential use as control (housekeeping) genes. A phenylalanine ammonia lyase (PAL) gene previously found to have relatively steady expression in pine xylem (Nairn et al. 2008) was tested for potential use as a housekeeping control but was found responsive to venom treatment. It was subsequently relegated to study as a candidate biomarker. Primers for qRT-PCR analysis of potential biomarker and control genes were designed using Primer 3 v.0.4.0 (Table 3.2).

Five-month-old loblolly pine seedlings (half-sib family of 7-56) were cut at the soil line and the ends were placed in 50 μ l of 20 mg/ml venom solution or water. After absorption of the treatment solution, seedlings were incubated in water. At intervals (4, 24, 48 h), seedlings were flash-frozen in liquid nitrogen. For testing of *P. radiata*, shoot tips collected from hedged trees were subjected to the same venom and control treatments as were used for loblolly seedlings. At 24 h, one venom-treated and one control shoot tip were flash-frozen, while the remaining three were incubated and observed for phenotypic responses over three weeks. Frozen tissues were

ground using a mortar and pestle, and RNA was extracted as described above. Complementary DNA was synthesized (as described previously but without modified nucleotides) and further diluted for PCR experiments.

Biomarker gene performance was initially assessed using semi-quantitative PCR as follows. For cDNAs derived from pine tissue samples, a two-fold dilution series from 0.25 ng/ μ l to 0.0078 ng/ μ l cDNA was generated as starting template material for PCR. Reaction mixtures contained 10 μ l of cDNA template in buffer, 3 μ l primer (0.5 μ M each), 4 μ l 10X buffer (100 mM Tris HCl pH 8.8, 35 mM MgCl₂, 250 mM KCl), 2 μ l 10 mM dNTP mix, and 1 μ l Taq polymerase. Reactions (50 μ l total volume) were run using a GeneAmp 9700 thermal cycler (Applied Biosystems, Carlsbad, CA). The amplification conditions were 95° C for 3 min, then 30 cycles of 94° C for 30 s, 55° C for 30 s, and 72° C for 90 s, followed by 72° C for 10 min. Amplification products were visualized in agarose gels for preliminary, qualitative assessment of differential expression of target genes. Amplimers were visualized by running 5 μ l of the PCR product solution on a 1.5% agarose gel in TAE buffer, followed by ethidium bromide staining.

For quantitative real-time PCR (qRT-PCR), reaction mixtures consisted of 5 μ l of 0.5 μ M primer pair, 5 μ l of 0.5 μ M cDNA template, and 10 μ l SybrGreen 2X Supermix (BioRad, Hercules, CA). Twenty microliter reactions were run using an iCycler thermal cycler (BioRad, Hercules, CA), and reaction conditions were 95° C for 3 min, then 40 cycles of 95° C for 30 s, 65° C for 45 s, and 78° C for 20 s, followed by 95° C for 1 min, 55° C for 1 min, and 81 cycles of 55° C for 10 s in 0.5° C increments to assess product purity via melt temperature. Samples were analyzed in triplicate for each primer set, and runs were considered acceptable when replicates agreed to <0.5% RSD. Data was generated from two biological replicates and two

technical replicates. Means from technical replicates were taken, and then used to calculate mean and error from the two biological replicates.

3.2.4 Proteolysis

To determine whether the woodwasp venom active factor had a proteinaceous component, venom was subjected to proteolysis. Proteinase K (110 μ l, 1 mg/ml in 50 mM Tris, pH 8.0, 10 mM CaCl₂) was added to 1 ml venom (20 mg/ml in water). A control digestion consisted of prepared Proteinase K that had been deactivated by boiling for 15 min. Both solutions were placed in a 45° C bath for 24 h, after which they were boiled to deactivate proteinase activity (venom activity is heat-stable), followed by centrifugation for 10 min at 17,000 g. Shoot tips from *P. radiata* trees previously characterized as either highly responsive or weakly responsive were treated with venom incubated with heat- inactivated Proteinase K, venom incubated with active Proteinase K, or a water control. For each treatment, 50 μ l of solution were absorbed by each of five shoot tips after which the shoot tips were transferred to pure water for further incubation. After 24 h, the shoot tips were pooled by treatment, flash-frozen in liquid nitrogen, RNA was extracted, and cDNA was synthesized as described previously. This material was then analyzed in the qRT-PCR assay described previously.

3.3 Results

3.3.1 Phenotypic assays

Drawing on literature reports, a variety of tissue explants, including needles, stems, callus and seedlings from different pine species, were tested initially for observable phenotypic responses to *S. noctilio* venom application. Seedlings (4-12 months old) cut at the soil line and allowed to absorb an aqueous dilution of venom displayed a relatively consistent phenotypic

response with loss of turgor pressure, concomitant needle droop, and a change in needle color to yellow-green or gray. Control and responsive seedlings displaying a clear phenotype are shown in Figure 3.1. *Pinus radiata* seedlings were observed, in general, to respond more quickly and with greater sensitivity to venom application than did *P. taeda* seedlings.

Unfortunately, in most experiments, a large proportion of venom-treated seedlings failed to display clear visual symptoms (escapes). In cases where seedlings gave visually observable responses, symptoms such as needle droop had response times ranging from two hours to two weeks. Color changes, when observed at all, typically only became noticeable after seven days and sometimes took up to three weeks to develop fully. On the other hand, no control seedlings were ever seen to display the phenotypic changes associated with venom application. The large proportion of escapes in assays using seedlings led us to consider that genetic differences between species and among individual genotypes could be confounding bioassays based on observable phenotypic changes.

3.3.2 Microarray analysis

Changes in the expression levels of genes responsive to biotic and abiotic agents can be measured quickly and with great sensitivity, which is the rationale for developing biomarker gene assays to supplant phenotypic assays (28-30). A time-series microarray experiment using the PtGen2 loblolly pine cDNA microarray was used to identify candidate biomarker genes that could be used in routine assays of pine tissue responses to *S. noctilio* venom. At a cutoff of 1.5-fold, a total of 885 genes showed altered expression in response to venom treatment. Table 3.1 lists the seven loblolly pine seedling genes whose expression was found by microarray analysis to be most increased at 24 and 48 h post-treatment with venom in comparison to controls. Three of these genes – those annotated as encoding a potential pathogenesis-related class 4 protein

(PR4) protein, a thaumatin-like protein (TLP), and an unknown protein – were chosen for further testing as potential biomarkers.

3.3.3 Biomarker validation

Quantitative real-time PCR, the method of choice for making rapid and sensitive measurements of changes in expression levels for individual genes (31), was used to validate results obtained from the microarray study. To develop a qRT-PCR-based biomarker assay, gene-specific oligonucleotide primers were generated for the three venom-sensitive genes identified in the microarray study. Primers were also designed for three additional genes, two actin genes (ACT1, ACT2) and one PAL gene, identified as useful control (housekeeping) genes in a previous study (32). The complete list of genes used in the qRT-PCR studies and their respective primer sequences are found in Table 3.2. Preliminary testing showed that all primer pairs yielded single amplicon products when using cDNA templates from either *P. taeda* or *P. radiata*.

To take advantage of the greater sensitivity of *P. radiata* to *S. noctilio* venom and at the same time investigate whether genotypic variation contributed to bioassay escapes, 17 *P. radiata* seedlings were pruned over several months to create hedges as a source of multiple shoot tips that could be used in replicated experiments. Shoot-tip bioassays subsequently demonstrated that phenotypic responses to venom did vary with respect to genotype and that the variation in response was consistent over several months (data not shown). Two trees graded as most responsive and one tree graded as non-responsive were chosen for subsequent use in semi-quantitative PCR and qRT-PCR assays to validate the biomarker genes.

Semi-quantitative PCR was used for initial assessment of the candidate biomarker genes identified in the microarray experiment. Of the six genes tested, the expression levels of four

were increased in *P. radiata* shoot tips treated with venom (Fig. 3.2). All three candidate biomarker genes – the putative PR4 protein, the putative TLP, and the unknown protein – as well as the PAL gene, responded to venom treatment. Both of the other candidate control genes (ACT1 and ACT2) showed little or no response to venom exposure. The PAL gene, which showed more consistent response to venom than the unknown gene, was substituted for the unknown gene as a biomarker in subsequent qRT-PCR analyses. A retrospective review of the microarray data noted 17 features on the array annotated as PAL, of which seven were differentially expressed in response to venom - five were up-regulated about two-fold, and two were down-regulated about the same amount.

In qRT-PCR assays using *P. taeda* seedlings, the strongest and most consistent responses were from the putative PR4 and TLP genes (Fig. 3.3). Response of the PR4 gene was more sensitive to *S. noctilio* venom exposure, while the TLP gene response was more consistent. PAL gene expression was consistently elevated in response to venom exposure, but to a lesser extent than either the PR4 or TLP genes. *Pinus radiata* shoot tips treated with venom showed the same general pattern of induced response for the PR4, TLP, and PAL biomarker genes (Fig. 3.4). Technical replicates of the same biological samples gave consistent results (data not shown).

3.3.4 Species and genetic differences in response to venom

Considerable differences in the degree of biomarker gene responsiveness to *S. noctilio* venom were observed between *P. taeda* and *P. radiata*. The increases in PR4, TLP, and PAL expression were always much greater in *P. radiata* tissues compared with similarly treated *P. taeda* tissues. In a representative comparison of *P. radiata* shoot tip and *P. taeda* seedling responses the differences in expression of the PR4 and TLP genes were on the order of 40- to 50-fold (Fig. 3.5). Hedged *P. radiata* trees provided a means to test over time using multiple

biological replicates whether individual pine genotypes had differential sensitivity to *S. noctilio* venom. As shown in Figure 3.6, there was a nearly 10-fold difference in the response levels of the *P. radiata* PR4 and TLP genes between trees that had been phenotypically evaluated as either venom-sensitive or non-responsive.

3.3.5 The effect of proteolysis on venom activity

Hymenopteran venoms typically contain a variety of bioactive proteins, peptides, and metabolites. To test whether the active factor in *S. noctilio* venom was proteinaceous, *P. radiata* shoot tips were treated with venom that had been incubated with either active or heat-inactivated Proteinase K. As typified by results shown in Figure 3.7, proteolytic treatment of *S. noctilio* venom consistently reduced the induction of biomarker gene expression in challenged tissues.

3.4 Discussion

Several researchers have in the past used pine tissue explants in bioassays to gauge the response of pines to attack by *S. noctilio* (10,12,13,15-18). Early in our studies, several phenotypic assays were tested with variable success. The bioassay developed here, based on gene expression response, is significantly faster, more sensitive, and more reproducible than previous bioassays based on visual observations or metabolic measures.

Whole-seedling bioassays were used to survey across pine genotypes for phenotypes indicating responsiveness to *S. noctilio* venom. Initially, *P. taeda* seedlings grown from open-pollinated orchard seed were used, and these were replaced with half-sib 7-56 *P. taeda* seedlings in an effort to reduce the number of escapes and avoid non-responsive genotypes. Finally, *P. radiata* seedlings were chosen for routine assays because they gave a stronger, more consistent phenotype, and were the species most widely utilized in the early *S. noctilio* literature.

Nonetheless, even using *P. radiata*, the phenotypic bioassays continued to yield variable results. Frequently, no more than 40% of the seedlings exposed to *S. noctilio* venom displayed obvious phenotypic responses: loss of needle turgor, growing tip droop, and/or chlorosis and browning of needles. In order to develop a more sensitive and consistent bioassay, gene expression in whole *P. taeda* seedlings exposed to venom was assessed using the PtGen2 microarray.

Explants from hedged trees did not decrease the number of escapes for phenotype assays; thus, up to 60% of the shoot tips from either *P. taeda* and *P. radiata* failed to respond, even when taken from the same plant. However, testing of multiple shoots from hedged trees ultimately identified individual trees that were more sensitive or less sensitive to woodwasp venom. These results were used to inform choices of specific trees for development of quantitative assays.

3.4.1 Gene expression assays

Previous studies have used microarrays to interrogate tree transcription-level responses to insect attack (33-36). While these studies focused on insect salivary secretions or mechanical wounding through herbivory as plant response elicitors, we have focused instead on a hymenopteran venom gland secretion introduced through oviposition as an inducer of pine transcriptional changes. The PtGen2 microarray experiments identified numerous pine genes that responded to woodwasp venom. Of the genes that responded most strongly at 24 and 48 h post-exposure, only those that were up-regulated more than 3-fold compared to control tissues were considered as potential biomarkers. Two of the three biomarker genes selected for testing (PR4 and TLP) have been described in many previous studies as being up-regulated in response to many biotic and abiotic stressors, including drought and various pests (37-42). While there is no evidence that the response of either gene is specific for woodwasp venom, they are clearly up-

regulated by venom compared to seedlings not exposed to venom and are, thus, of use for following the effects of venom or its components in various tissues and under varying conditions. The responses of these genes were validated using both semi-quantitative and quantitative PCR assays. While visually-assessed phenotypic assays of venom-treated seedlings routinely suffered rates of escape as high as 40%, pine seedlings or tissues treated with venom always demonstrated increased transcription of the PR4 and TLP biomarkers relative to controls, even when physical symptom were absent.

Semi-quantitative PCR analyses showed that PR4, TLP, UNK, and PAL were all strongly induced by woodwasp venom, and that ACT1 and ACT2 were suitable as control genes (Fig. 3.2). The results were consistent across both *P. taeda* and *P. radiata*. Previous work has shown both PR-4 and thaumatin-like proteins (TLPs) to be induced as part of the water stress response in a wide variety of plant species (38,43). Up-regulation of these genes in pine tissues responding to woodwasp venom is consistent with early studies of the *Pinus-Sirex* pathosystem that hypothesized altered water relations in pine tissues (9,44,45). Although the PAL gene had been suggested as a potential housekeeping gene on the basis of its steady expression in pine stem tissues, its weak induction by woodwasp venom was not a complete surprise. Induction of phenylpropanoid pathway genes, including PAL, is a common plant response to a variety of biotic and abiotic stresses (46).

3.4.2 Interspecific and intraspecific variation of venom response

Variations in response to *S. noctilio* venom among pine species has been reported previously. Spradbery measured leaf pressure changes among 29 *Pinus* species as a phenotypic response to woodwasp venom (12). However, this report is the first to quantitatively measure gene expression-level responses to woodwasp venom in different species. In our work, *P. radiata*

and *P. taeda* clearly showed different trajectories of response to woodwasp venom, and based on 24-h exposure times, *P. radiata* explants responded more strongly to woodwasp venom than did those from *P. taeda*. Genotypic variations in response to woodwasp venom were suggested from the earliest studies of this pathosystem (10). Differences in response to *S. noctilio* venom between genotypes within a single pine species are quantified here for the first time. We have shown that such tree-to-tree differences are manifest at the level of gene expression in both *P. taeda* and *P. radiata*, and we observed that variation across pine species and genotypes, while similar, varied quantitatively.

Demonstration of significant differences in response to woodwasp venom between pine species and individual genotypes allows for practical screening of native pines for venom responsiveness. Such information will be useful for modifying risk maps for *Sirex* invasion as the species moves southward in the U.S. Application of this screening to individual genotypes may prove a valuable tool in breeding pines resistant to damage by *S. noctilio* attack. However, it remains to be determined whether a strong response to venom correlates with overall resistance or susceptibility and leads to tree mortality. A clear understanding of this relationship will facilitate both risk map reassessment and establishment of practical breeding programs.

3.4.3 Biochemical nature of active factor(s)

Hymenopteran venoms have been shown to contain high molecular weight enzymes and allergenic proteins (phospholipase, hyaluronidase, proteases), as well as low molecular weight entities, such as peptides and kinins (apamin, bradykinin, mastoparan), small amines (histamine, serotonin), protease inhibitors (serpins), and neurotoxins (47-53). However, relatively little is known about the composition of *S. noctilio* venom. Boros (54) characterized *S. noctilio* bulk venom as an acid-mucopolysaccharide-protein complex, which was similar to reports for closely

related endoparasitoid hymenopteran venoms that commonly contain acidic, often glycosylated proteins (55). Previous characterization efforts left open the question of whether or not the principal active agent in *S. noctilio* venom was proteinaceous (Wong and Crowden 1976). There was a clear diminution of PR4 gene induction in both highly responsive and weakly-responsive trees treated with intact versus proteolyzed venom. A similar pattern was seen for the expression of the TLP biomarker as well (data not shown). That there were quantitative differences in the magnitude of the gene responses between the different tree genotypes assayed could indicate the presence of more than one active component in venom measured by this assay. Nonetheless, attenuation of the PR4 expression response was consistent among the genotypes tested, clearly indicating that a proteinaceous component in the venom is an important elicitor of the response. This study is the first quantitative examination of the biochemical identity of the active factors in the venom. Work is underway to define the exact biochemical nature of the active component(s) in *S. noctilio* venom.

The bioassay we have developed will facilitate identification of the specific active factor(s) in *S. noctilio* venom. With the purified factor(s) in hand we will be able to explore their mechanisms of action as well as their targets in pine tissues. Findings from those efforts should enable development of new protective measures, including the breeding of trees with enhanced resistance against *S. noctilio* attack.

3.5 Conclusions

Understanding of the gene-level responses of pines to an introduced pathosystem may allow researchers to identify resistant genotypes of valuable species and include them in breeding programs. The present work has identified several pine genes that respond quickly and

strongly to woodwasp venom exposure. Gene-expression assays based on these biomarkers will facilitate molecular dissection of the venom as well as the host response and provide the information on which to base directed breeding programs. Eventual purification of specific active factor(s) from the venom will give new insight into the some of the mechanisms by which insects manipulate host-plant defense responses in order to facilitate infection by damaging plant pathogens.

Funding

The authors wish to acknowledge support from the USDA/CSREES McIntire-Stennis Program (GEOZ-0154-MS) as well as assistantship funding to J.M.B. from the Warnell School of Forestry and Natural Resources.

Acknowledgments

The authors thank Kelley Zylstra (USDA-APHIS, Syracuse, NY) for providing *Sirex noctilio* wasps, Dr. Campbell Nairn for actin and PAL qRT-PCR primer sequences, and Stephen Pettis for greenhouse care of *P. radiata* hedges.

Table 3.1: Genes identified in *P. taeda* seedlings showing the greatest fold-expression increases (log₂ normalized) at 24 and 48 h post-treatment with *S. noctilio* venom. Genes noted in boldface were selected for initial development of PCR assays.

Gene ID	Fold change 24 hr	Fold change 48 hr	Functional Category	Clone Name	NCBI NR BLASTx	NCBI NR Annotation
11.9.14	3.0	4.0	Defense	NDL1_35_C04_A029	gb AAC33732.1	PR-4 type protein (PR4) [<i>Vitis vinifera</i>]
7.15.6	5.8	3.3	Defense	FLD1_38_F11_A029	ref XP_002275386.1	class IV chitinase [<i>Vitis vinifera</i>], predicted
61.19.9	2.7	2.8	Defense	STRR1_17_E07_A033	emb CAA10492.1	Thaumatococcus-like protein (TLP) [<i>Pseudotsuga menziesii</i>]
43.20.7	2.9	2.7	unknown	RTCA1_29_F06_A029	not found	unknown (UNK)
23.4.12	4.9	2.7	unknown	RTDS1_10_C05_A015	not found	unknown
43.23.10	4.1	2.6	Defense	RTCNT1_8_B10_A029	dbj BAD93486.1	pollen allergen CJP38 [<i>Cryptomeria japonica</i>]
63.12.9	4.8	2.5	Defense	STRR1_57_A10_A033	gb ABY66958.1	class IV chitinase [<i>Vitis pseudoreticulata</i>]

Table 3.2: Gene annotations and primer sequences used in PCR assays.

Gene name	Primer Sequence (5'→3')
<i>PR4</i> (PR-4 protein, <i>Vitis vinifera</i>)	5': TTA TAG GCG GAC GGG TTG TA 3': GTT TCA GCA CAA ACG CAG AG
<i>TLP</i> (Thaumatococin-like protein, <i>Pseudotsuga menziesii</i>)	5': GTT TGC ACC AGA AGC ACA TG 3': CAC AGG CCT ACA GCT ATG CA
UNK (Unknown, <i>Pinus taeda</i>)	5': CCA TGC ATC CAC CAA CAG TA 3': GGA TGA GGC CGG GAA TAT A
<i>PAL1</i> (Phenylalanine ammonia lyase, <i>Pinus taeda</i>)	5': TC ACA AGC TGA AGC ACC ATC 3': T CCC GTC CAA GAC ATA CTC C
<i>ACT1</i> (Actin, <i>Pinus taeda</i>)	5': AA TGG TCA AGG CTG GAT TTG 3': AG GGC GAC CAA CAA TAC TTG
<i>ACT2</i> (Actin, <i>Pinus taeda</i>)	5': TT GCT GAC CGT ATG AGC AAG 3': GA GGT GCA ACC ACC TTG ATT

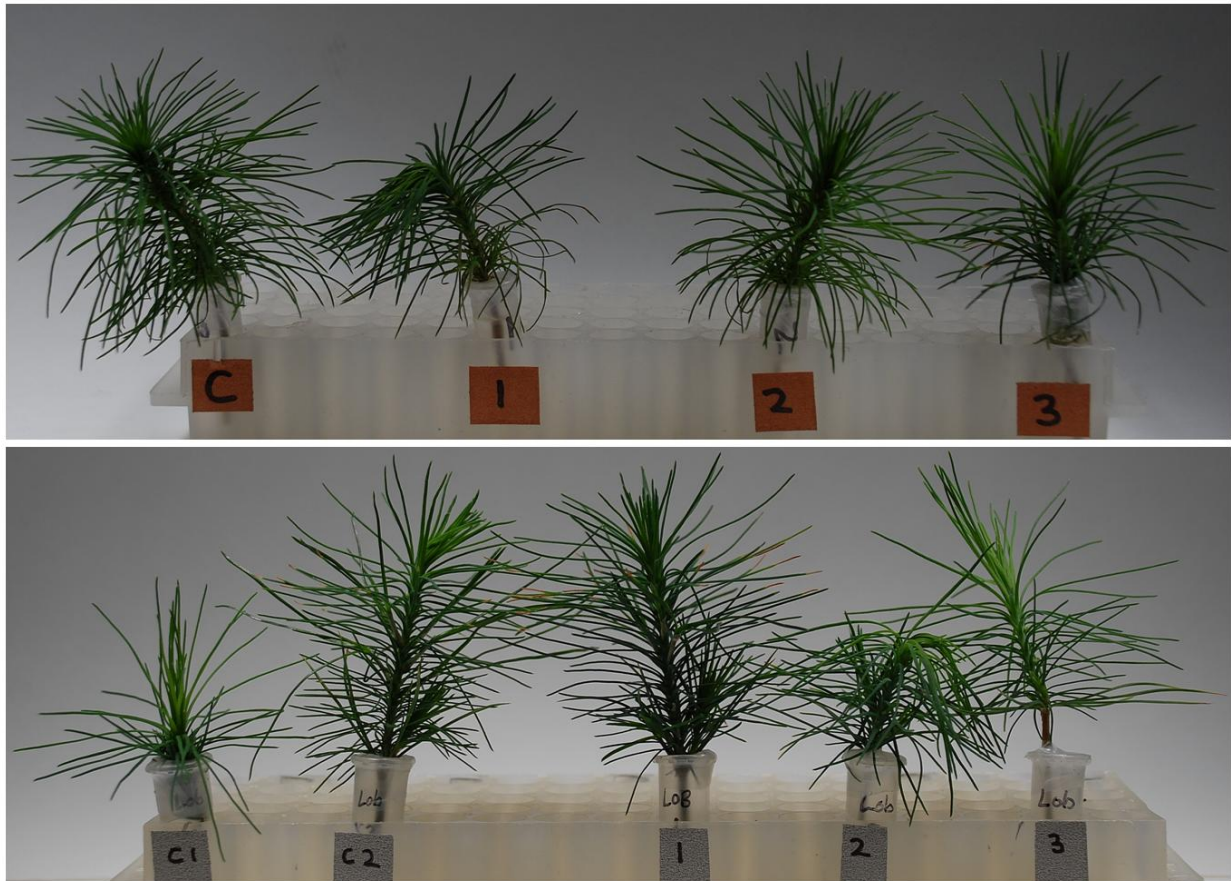


Figure 3.1: Typical phenotypic responses in pine seedlings treated with *S. noctilio* venom. Four-month-old pine seedlings were cut at the soil line and placed into an aqueous solution of *S. noctilio* venom or water until absorbed and then transferred to deionized water. Top, *P. radiata*, seven days after treatment. C is a water control; 2 and 3 are venom-treated escapes. Bottom, *P. taeda*, first day of treatment. C1 and C2 are water controls; 1 and 3 are escapes.

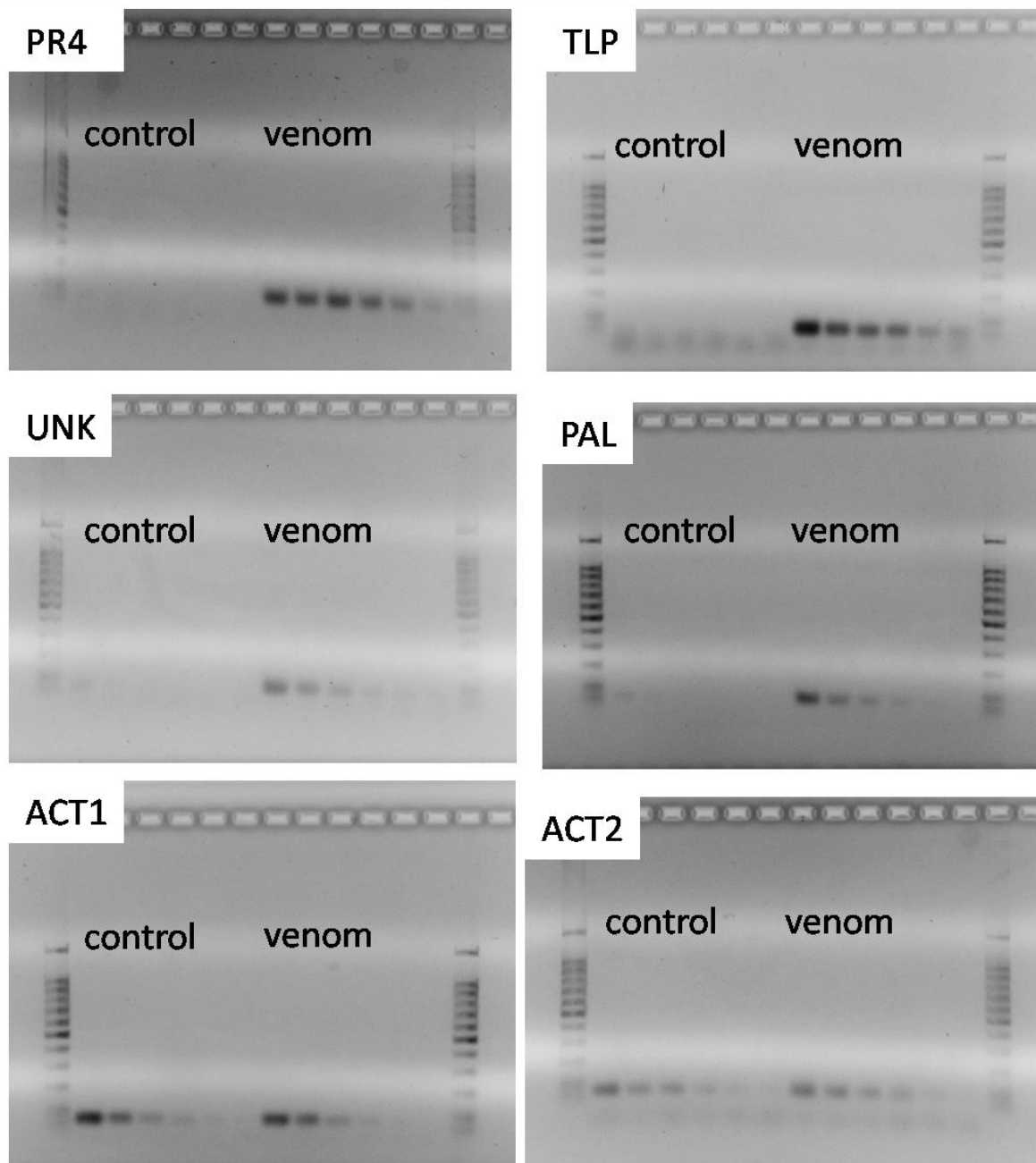


Figure 3.2: Semi-quantitative PCR analysis of pine gene response to *S. noctilio* venom. Cuttings from a *P. radiata* tree previously demonstrated as sensitive to venom were exposed to 20 mg/ml *S. noctilio* venom and incubated 24 h. Amplified products from a dilution series of template cDNA (2.5 ng to 0.078 ng in lanes running left to right) were resolved by agarose gel electrophoresis and visualized by staining with ethidium bromide. PR4, PR-4 protein; TLP, thaumatin-like protein; UNK, unknown pine gene product upregulated by *S. noctilio* venom; PAL, phenylalanine ammonia lyase; ACT1, ACT2, actins. Template dilutions from cuttings treated with water only (controls) are shown in six lanes on the left in each panel, while dilutions from cuttings treated with venom are in six lanes on the right. Sample lanes in each gel are flanked by 100 kb ladders.

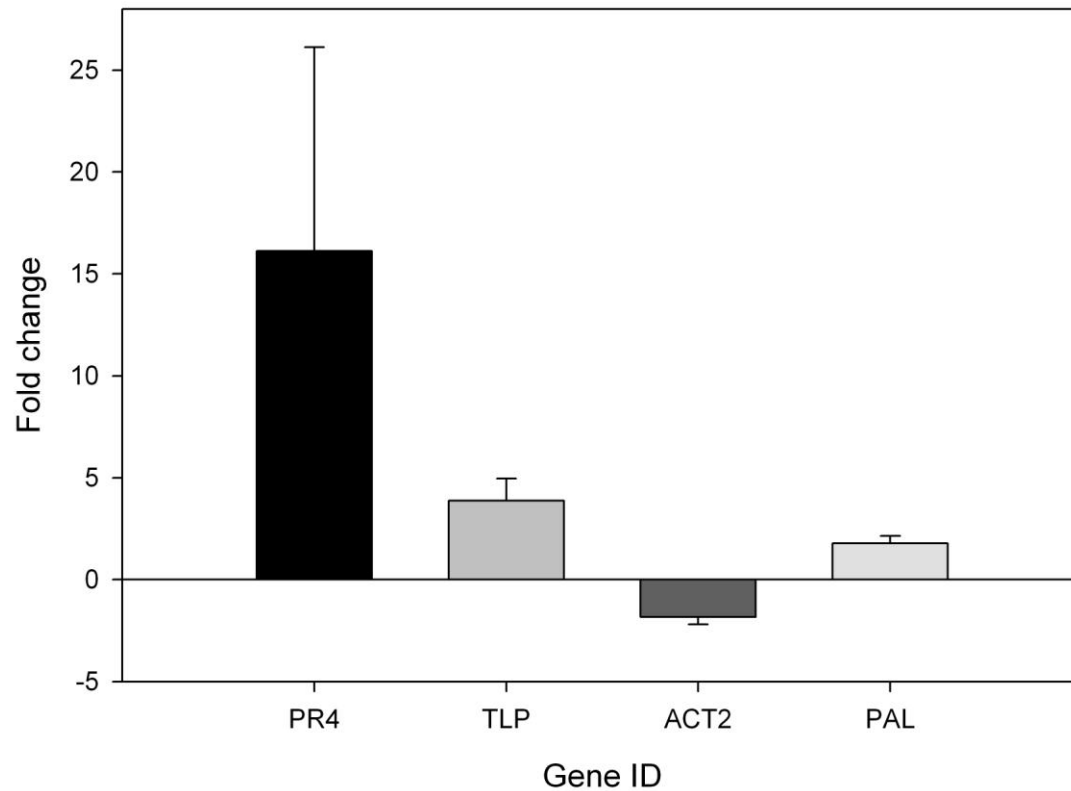


Figure 3.3: *Pinus taeda* gene responses to *S. noctilio* venom. RNA (from *P. taeda* seedlings incubated for 24 h after venom treatment) was used to prepare cDNA templates for qRT-PCR analyses. Fold changes are reported as the average of two biological replicates measured in three technical replicates (n=2). Values less than one are represented as negative numbers to show suppressed expression relative to the ACT1 control. Fold changes are normalized to water controls run for each sample.

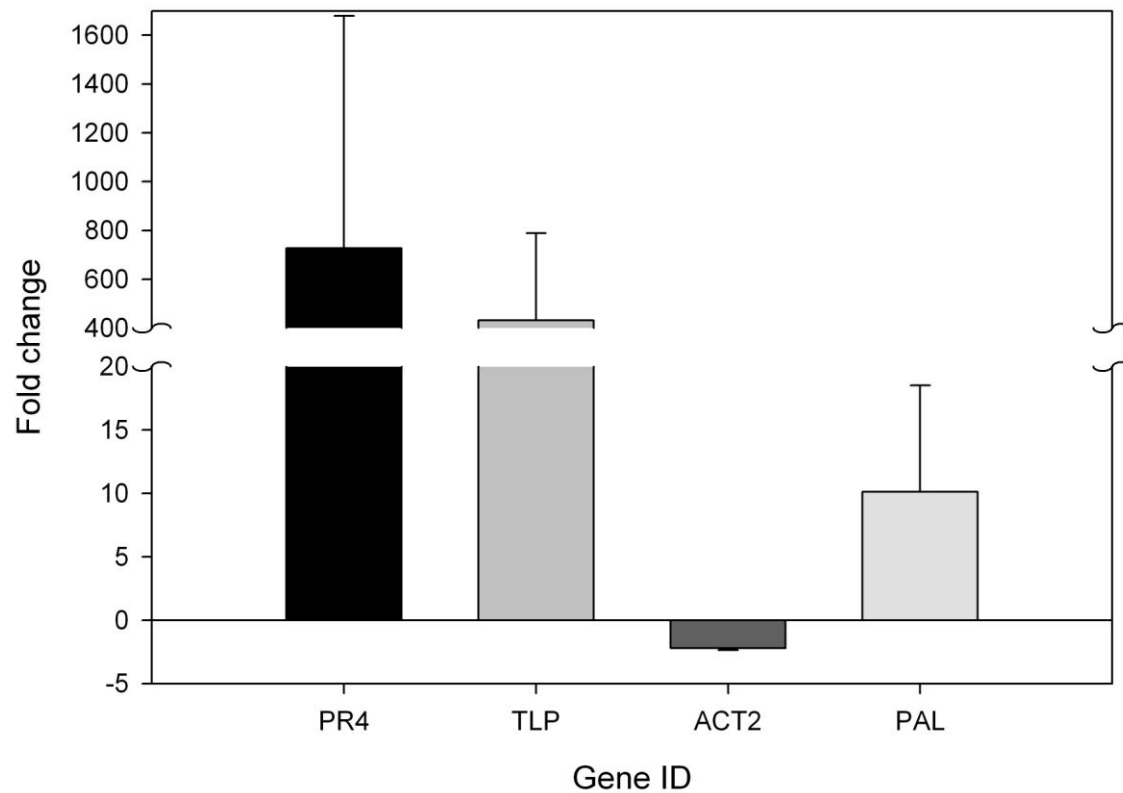


Figure 3.4: *Pinus radiata* gene responses to *S. noctilio* venom. RNA from *P. radiata* shoot tips incubated for 24 h after venom exposure was used to prepare cDNA templates for qRT-PCR analyses. Fold changes are reported as the average of two biological replicates measured in three technical replicates (n=2). Values less than one are represented as negative numbers to show suppressed expression relative to the ACT1 control. Fold changes are normalized to water controls run for each sample.

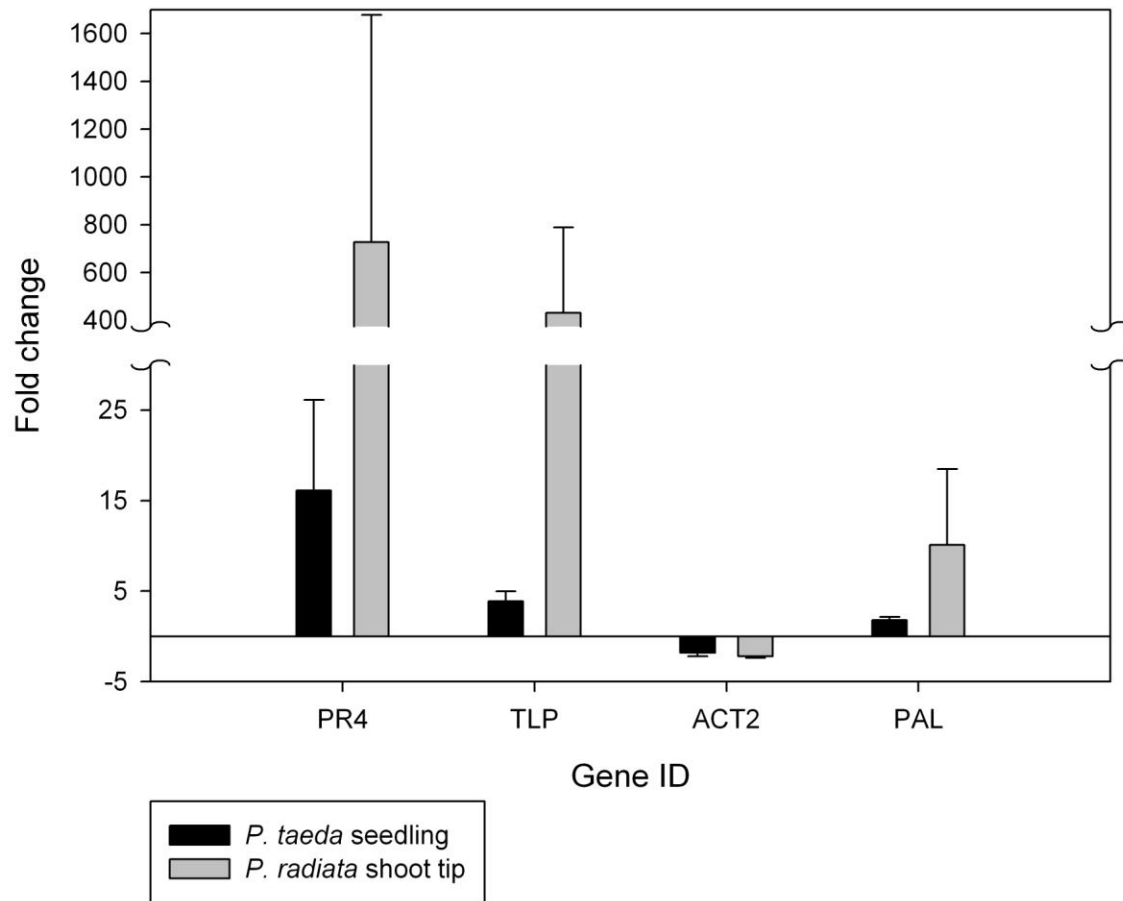


Figure 3.5: Gene expression-level differences between pine species in response to woodwasp venom exposure. Quantitative PCR bioassay measured biomarker gene responses to *S. noctilio* woodwasp venom. *Pinus taeda* whole seedlings treated with *S. noctilio* venom are compared with *P. radiata* shoot tips treated with venom. Both were sampled 24 h after exposure. Values less than one are represented as negative numbers to show suppressed expression relative to a control. Fold changes are normalized to water controls run for each sample.

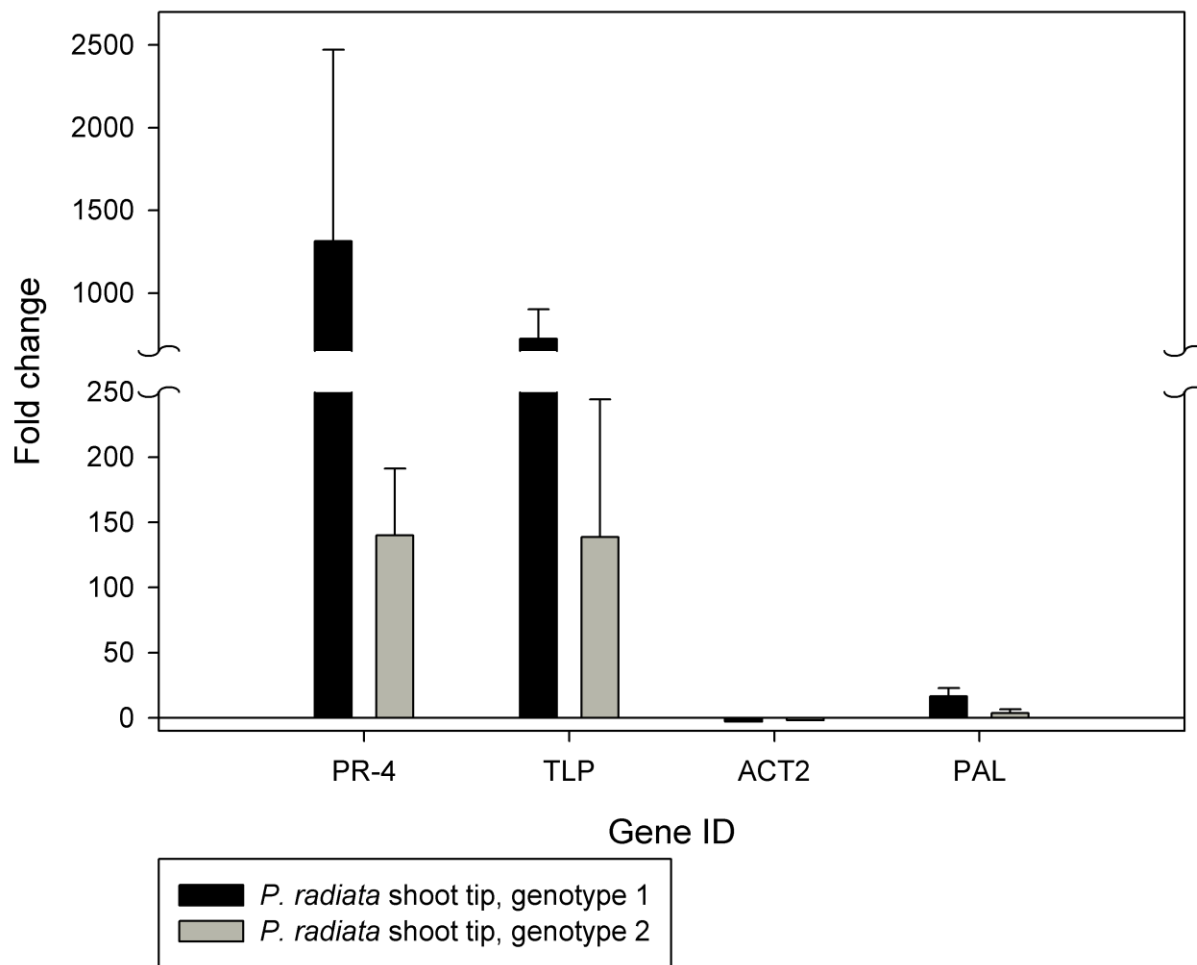


Figure 3.6: Within-species variation in *P. radiata* biomarker gene response to *S. noctilio* venom. Shoot tips from hedged seedlings were compared for this test 24 h after venom exposure, using qRT-PCR as the bioassay. Values less than one are represented as negative numbers to show suppressed expression relative to a control. Fold changes are normalized to water controls run for each sample.

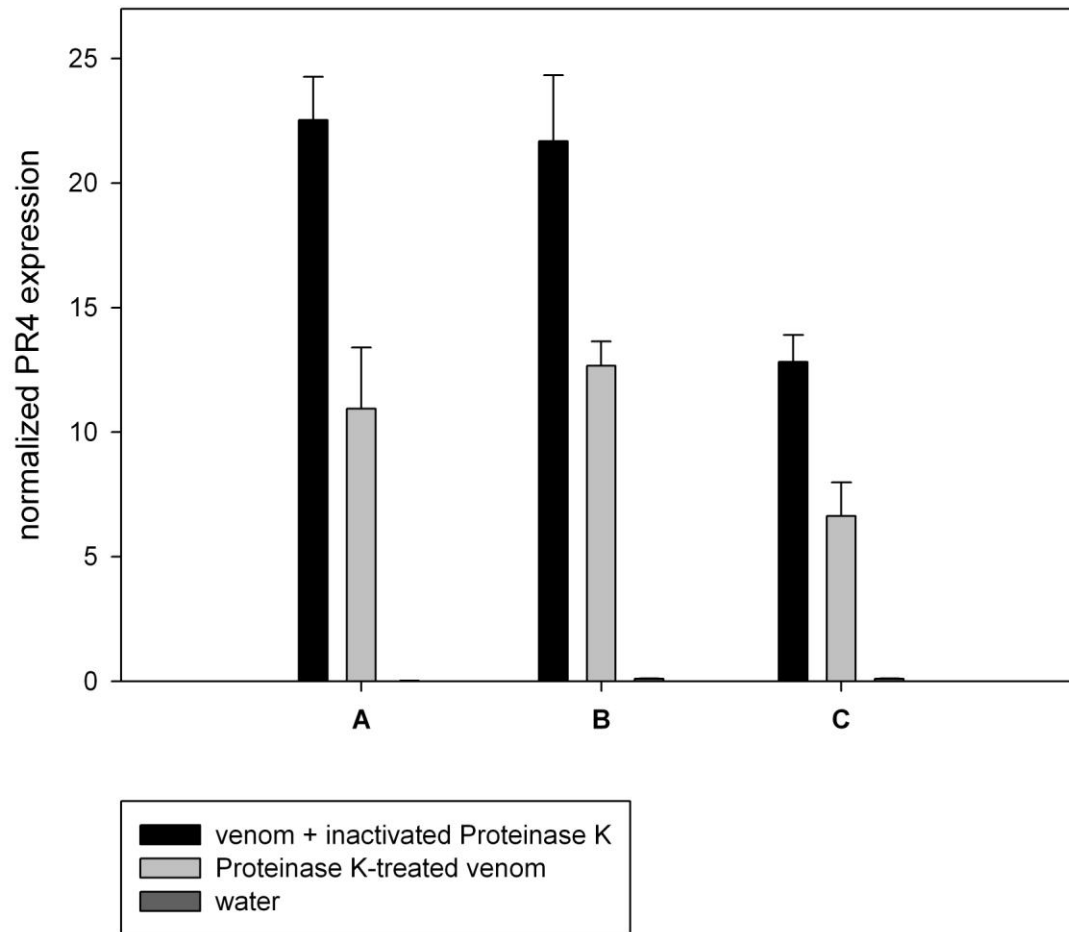


Figure 3.7: The effect of proteolysis on induction of biomarker genes by *S. noctilio* venom. Shoot tips from two *P. radiata* trees (A and B) previously identified as highly responsive to venom, and one tree (C) identified as weakly responsive to venom were challenged with venom that was incubated with either active Proteinase K or heat-inactivated Proteinase K or with a water blank. Response of the PR4 biomarker gene is displayed as PR4 expression normalized to the ACT1 control gene.

References

1. Borchert, D., Fowler, G., and Jackson, L. (2007) Organism pest risk analysis: risk to the conterminous United States associated with the woodwasp, *Sirex noctilio* Fabricius, and the symbiotic fungus, *Amylostereum areolatum* (Fries: Fries) Boidin. USDA-APHIS-PPQ-EDP, Raleigh, NC USA
2. Dunkle, R. L. (2005) Detection of the European wood wasp, *Sirex noctilio* (Fabricius) in New York. USDA, APHIS
3. Dunkle, R. L. (2006) Detection of *Sirex noctilio* Fabricius (Hymenoptera: Siricidae) (Sirex woodwasp) in Allegany, Erie, Genesee, Jefferson, Livingston, Monroe, Niagara, Oneida, Ontario, Orleans, Schuyler, Wyoming and Yates counties in New York. USDA-APHIS
4. Dunkle, R. L. (2006) Detection of *Sirex noctilio* Fabricius (Sirex woodwasp) in Tioga County, Pennsylvania. USDA-APHIS
5. Borchert, D., Fowler, G., and Jackson, L. (2006) Organism pest risk analysis: risk to the conterminous United States associated with the woodwasp, *Sirex noctilio* Fabricius, and the symbiotic fungus, *Amylostereum areolatum* (Fries: Fries) Boidin. USDA-APHIS-PPQ-EDP, Raleigh, NC USA
6. Coutts, M. P. D., J. E. (1966) Polyphenols and resin in the resistance mechanism of *Pinus radiata* attacked by the wood wasp, *Sirex noctilio*, and its associated fungus. (Department of National Development, F. a. T. B. ed., Commonwealth of Australia
7. Ciesla, W. M. (2003) European Woodwasp: A Potential Threat to North Americas Conifer Forests. *Journal of Forestry* **101**, 18-23

8. Talbot, P. H. B. (1977) *Sirex-Amylostereum-Pinus* association. *Annual Review of Phytopathology* **15**, 41-54
9. Coutts, M. P. (1968) Rapid physiological change in *Pinus radiata* following attack by *Sirex noctilio* and its associated fungus, *Amylostereum* sp. *Aust. J. Sci.* **30**, 275-277
10. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .2. Effects of *S. noctilio* mucus. *Aust. J. Biol. Sci.* **22**, 1153-1161
11. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .I. Effects of symbiotic fungus *Amylostereum* sp. (Thelophoraceae). *Aust. J. Biol. Sci.* **22**, 915-924
12. Spradbery, J. P. (1973) A comparative study of the phytotoxic effects of siricid woodwasps on conifers. *Ann. Appl. Biol.* **75**, 309-320
13. Coutts, M. P. (1970) The physiological effects of the mucus secretion of *Sirex noctilio* on *Pinus radiata*. *Aust. For. Res.* **4**, 23-26
14. Bordeaux, J. M., and Dean, J. F. D. (2012) Susceptibility and Response of Pines to *Sirex noctilio*. in *The Sirex Woodwasp and its Fungal Symbiont*: (Slippers, B., de Groot, P., and Wingfield, M. J. eds.), Springer Netherlands. pp 31-50
15. Fong, L. K., and Crowden, R. K. (1973) Physiological effects of mucus from wood wasp, *Sirex noctilio* F., on foliage of *Pinus radiata* D. Don. *Aust. J. Biol. Sci.* **26**, 365-378
16. Wong, L. K., and Crowden, R. K. (1976) Preliminary studies on mucus secretion of wood wasp, *Sirex noctilio* F .1. Physicochemical and biochemical properties. *Aust. J. Biol. Sci.* **29**, 21-32

17. Kile, G. A., Bowling, P. J., Dolezal, J. E., and Bird, T. (1974) The reaction of *Pinus radiata* twigs to the mucus of *Sirex noctilio* in relation to resistance to *Sirex* attack. *Aust. For. Res.* **6**, 25-34
18. Bowling, J. P., and Dolezal, J. E. (1970) Initial note on the development of a mucus test to determine resistance of *Pinus radiata* to attack by *Sirex noctilio*. *Aust. For. Res.* **5**, 57-62
19. Gullan, P. J., and Cranston, P. S. (2000) *The Insects : an Outline of Entomology*, 2nd ed., Blackwell Science, Malden, MA
20. P., B. J., and F., M. B. (1967) Germination of Loblolly Pine Seed Hastened by Soakings in Aerated Cold Water. *Tree Planter's Notes* **18**, 24-25
21. Kerr, M. K. (2003) Design considerations for efficient and effective microarray studies. *Biometrics* **59**, 822-828
22. Chang, S., Puryear, J., and Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* **11**, 113-116
23. Lorenz, W. W., Yu, Y.-S., and Dean, J. F. D. (2010) An improved method of RNA isolation from loblolly pine (*P. taeda* L.) and other conifer species. *J. Vis. Exp.* **36**
24. Alba, R., Payton, P., Fei, Z. J., McQuinn, R., Debbie, P., Martin, G. B., Tanksley, S. D., and Giovannoni, J. J. (2005) Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. *Plant Cell* **17**, 2954-2965
25. Lorenz, W., Yu, Y.-S., Simões, M., and Dean, J. (2009) Processing the Loblolly Pine PtGen2 cDNA Microarray. *Journal of Visualized Experiments* **25**

26. Simon, R., Lam, A., Li, M.-C., Ngan, M., Menenzes, S., and Zhao, Y. (2007) Analysis of gene expression data using BRB-Array Tools. *Cancer Informatics* **2007**
27. Wang, X. J., Hessner, M. J., Wu, Y., Pati, N., and Ghosh, S. (2003) Quantitative quality control in microarray experiments and the application in data filtering, normalization and false positive rate prediction. *Bioinformatics* **19**, 1341-1347
28. Zimmermann, P., Laule, O., Schmitz, J., Hruz, T., Bleuler, S., and Gruissem, W. (2008) Genevestigator transcriptome meta-analysis and biomarker search using rice and barley gene expression databases. *Mol. Plant.* **1**, 851-857
29. Jeong, S. W., and Seo, Y. R. (2011) Transcriptomic analysis of *Caenorhabditis elegans* exposed to nickel (II) acetate using microarray. *BioChip J.* **5**, 78-85
30. Kim, H., Watkinson, J., and Anastassiou, D. (2011) Biomarker Discovery Using Statistically Significant Gene Sets. *J. Comput. Biol.* **18**, 1329-1338
31. VanGuilder, H. D., Vrana, K. E., and Freeman, W. M. (2008) Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques* **44**, 619-626
32. Nairn, C. J., Lennon, D. M., Wood-Jones, A., Nairn, A. V., and Dean, J. F. D. (2008) Carbohydrate-related genes and cell wall biosynthesis in vascular tissues of loblolly pine (*Pinus taeda*). *Tree Physiol.* **28**, 1099-1110
33. Philippe, R. N., Ralph, S. G., Mansfield, S. D., and Bohlmann, J. (2010) Transcriptome profiles of hybrid poplar (*Populus trichocarpa* x *deltoides*) reveal rapid changes in undamaged, systemic sink leaves after simulated feeding by forest tent caterpillar (*Malacosoma disstria*). *New Phytologist* **188**, 787-802
34. Ralph, S. G., Yueh, H., Friedmann, M., Aeschliman, D., Zeznik, J. A., Nelson, C. C., Butterfield, Y. S. N., Kirkpatrick, R., Liu, J., Jones, S. J. M., Marra, M. A., Douglas, C.

- J., Ritland, K., and Bohlmann, J. (2006) Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant Cell Environ.* **29**, 1545-1570
35. Ralph, S., Park, J. Y., Bohlmann, J., and Mansfield, S. D. (2006) Dirigent proteins in conifer defense: gene discovery, phylogeny, and differential wound- and insect-induced expression of a family of DIR and DIR-like genes in spruce (*Picea* spp.). *Plant Molecular Biology* **60**, 21-40
36. Ralph, S. G., Jancsik, S., and Bohlmann, J. (2007) Dirigent proteins in conifer defense II: Extended gene discovery, phylogeny, and constitutive and stress-induced gene expression in spruce (*Picea* spp.). *Phytochemistry* **68**, 1975-1991
37. Jayaraj, J., Muthukrishnan, S., Liang, G. H., and Velazhahan, R. (2004) Jasmonic acid and salicylic acid induce accumulation of beta-1,3-glucanase and thaumatin-like proteins in wheat and enhance resistance against *Stagonospora nodorum*. *Biol. Plant.* **48**, 425-430
38. Liu, J. J., Sturrock, R., and Ekramoddoullah, A. K. M. (2010) The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function. *Plant Cell Reports* **29**, 419-436
39. Liu, J.-J., Zamani, A., and Ekramoddoullah, A. K. M. (2010) Expression profiling of a complex thaumatin-like protein family in western white pine. *Planta* **231**, 637-651
40. Bertini, L., Leonardi, L., Caporale, C., Tucci, M., Cascone, N., Di Berardino, I., Buonocore, V., and Caruso, C. (2003) Pathogen-responsive wheat PR4 genes are induced by activators of systemic acquired resistance and wounding. *Plant Sci.* **164**, 1067-1078

41. Chung, S.-Y., Lee, K.-A., Oh, K.-J., and Cho, T.-J. (2005) Molecular characterization of a PR4 gene in Chinese cabbage. *Integrative Biosciences* **9**, 239-244
42. Li, X., Xia, B., Jiang, Y., Wu, Q., Wang, C., He, L., Peng, F., and Wang, R. (2010) A new pathogenesis-related protein, LrPR4, from *Lycoris radiata*, and its antifungal activity against *Magnaporthe grisea*. *Molecular Biology Reports* **37**, 995-1001
43. Wang, N. L., Xiao, B. Z., and Xiong, L. Z. (2011) Identification of a cluster of PR4-like genes involved in stress responses in rice. *J. Plant Physiol.* **168**, 2212-2224
44. Madden, J. L. (1977) Physiological Reactions of *Pinus radiata* to Attack by Woodwasp, *Sirex noctilio* F. (Hymenoptera-Siricidae). *Bulletin of Entomological Research* **67**, 405-426
45. Madden, J. L., Coutts, M.P. (1979) The Role of Fungi in the Biology and Ecology of Woodwasps (Hymenoptera: Siricidae). in *Insect-fungus symbiosis : nutrition, mutualism, and commensalism : proceedings of a symposium* (Batra, L. R. ed.), Halsted Press, Montclair, NJ. pp 165-174
46. Dixon, R. A., and Paiva, N. L. (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell* **7**, 1085-1097
47. Piek, T., and Spanjer, W. (1986) *Chemistry and pharmacology of solitary wasp venoms*,
48. Hoffman, D. R. (2010) Ant venoms. *Curr. Opin. Allergy Clin. Immunol.* **10**, 342-346
49. Monteiro, M. C., Romao, P. R. T., and Soares, A. M. (2009) Pharmacological perspectives of wasp venom. *Protein Pept. Lett.* **16**, 944-952
50. Asgari, S., and Rivers, D. B. (2011) Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. in *Annual Review of Entomology, Vol 56* (Berenbaum, M. R., Carde, R. T., and Robinson, G. E. eds.), Annual Reviews, Palo Alto. pp 313-335

51. de Lima, P. R., and Brochetto-Braga, M. R. (2003) Hymenoptera venom review focusing on *Apis mellifera*. *J. Venom. Anim. Toxins* **9**, 149-162
52. Piek, T. (1990) Neurotoxic kinins from wasp and ant venoms. *Toxicon* **29**, 139-149
53. Colinet, D., Dubuffet, A., Cazes, D., Moreau, S., Drezen, J. M., and Poirie, M. (2009) A serpin from the parasitoid wasp *Leptopilina boulardi* targets the *Drosophila* phenoloxidase cascade. *Dev. Comp. Immunol.* **33**, 681-689
54. Boros, C. B. (1968) *The relationship between the woodwasp Sirex noctilio F. and the wood-rot fungus Amylostereum sp.* Master of Agricultural Science M. S., University of Adelaide
55. Asgari, S. (2006) Venom proteins from polydnavirus-producing endoparasitoids: their role in host-parasite interactions. *Arch. Insect Biochem. Physiol.* **61**, 146-156

CHAPTER 4

CHARACTERIZATION OF THE VENOM GLAND TRANSCRIPTOME FROM THE
WOODWASP, SIREX NOCTILIO F., IDENTIFIES HIGHLY EXPRESSED TRANSCRIPTS
ENCODING A LACCASE-LIKE MULTICOPPER OXIDASE AND OTHER PROTEINS
THAT MODULATE THE RESPONSE OF PINES TO INSECT ATTACK³

³ Bordeaux, J.M., W.W. Lorenz, S. Ayyampalayam, U. Bagal and J.F.D. Dean. Submitted to PLoS One, 01/21/2014.

Abstract

Sirex noctilio, the European horntail woodwasp, is an invasive insect that attacks conifer hosts (principally *Pinus* spp.) by ovipositing in the xylem, depositing eggs, fungal oidia, and a venom that causes rapid physiological changes in the host tree. To identify proteinaceous factors in the venom that affect pine defense responses, and in a broader effort to elucidate the underlying pathosystem generally, RNA isolated from the venom gland tissues of emergent female woodwasps was used to prepare cDNAs for short-read shotgun sequencing. The venom gland transcriptome was assembled from >260 million Illumina HiSeq reads using a minimum length cutoff for assemblies of 100 nucleotides. The resulting dataset, housed in a searchable database, is the first transcriptome-scale resource available for a species in a basal order of the Hymenoptera. Digital gene expression was employed to determine relative transcript abundance and to identify highly-expressed transcripts likely to encode venom components involved in targeting pine defense responses. Of the 68,887 transcripts generated from this dataset, 24,471 (35%) returned annotations with Expect value $\leq 1 \times 10^{-10}$. However, annotations were not returned for the seven most abundant transcripts. Significantly, a transcript encoding a putative laccase-like multicopper oxidase was the most highly-expressed, annotated sequence recovered. Similar to other hymenopteran venom transcriptomes, transcripts encoding putative esterases, lipases, phosphatases, and proteases were highly-expressed in the *S. noctilio* venom tissue. Transcripts were examined for evidence of *Sirex* affiliates and sequences belonging to nematodes in the genus *Deladenus* were identified. The transcriptome assembled from this study should find utility in identifying molecular players in the *S. noctilio*-*Pinus* pathosystem, as well as broad applicability in hymenopteran comparative genomics and venomics.

4.1 Introduction

Non-native forest insects are estimated to cause more than \$1.7 billion in property value and timber loss and \$2.3 billion in total government expenditures within the continental U.S. each year (1). *Sirex noctilio*, the European woodwasp, which attacks a broad range of pine species and is a serious pest on North American species grown in commercial plantations in the southern hemisphere, has recently become established in the eastern U.S. and Canada (2,3).

Sirex noctilio Fabricius is a Eurasian horntail woodwasp of the order Hymenoptera, suborder Symphyta, superfamily Siricoidea, family Siricidae (4). The Siricoidea, together with six other superfamilies, comprise the paraphyletic suborder Symphyta (*symphyta*, grown together, referring to absence of the wasp waist), which is frequently generalized as the sawflies and woodwasps. They are a basal suborder of the Hymenoptera, distinct from the Apocrita (*apokritos*, separated, also a reference to body sectioning) that are the most familiar members of this order. An important distinction between these Hymenopteran branches is their respective development and use of ovipositors. The Symphyta use their ovipositors primarily to deposit eggs, but as in the case of woodwasps, may also use them to deliver glandular secretions and other materials into host plants. With respect to their ovipositors, the Apocrita may be further distinguished into the Terebrantia (*terebra*, “drill”), parasitoids that use their ovipositors to deliver both eggs and venom into hosts, and the Aculeata (*aculeus*, “sting”), insects in which the ovipositor has been modified to deliver only venom to targets such as predators. Within Apocrita, seven ants, one bee, and three species in the parasitoid wasp genus *Nasonia* (Terebrantia) have had their genomes fully sequenced (5-12). Among these, the *Nasonia* species are the closest relatives of *S. noctilio*. No other hymenopteran species currently have fully-sequenced genomes.

Briefly, the *S. noctilio* life-cycle begins with oviposition, which also constitutes the start of the process that leads to tree mortality (13). Females drill one to five small holes through the bark of a prospective host and into the underlying xylem tissues. Each hole receives an injection of a viscous mixture of secretions from reproductive tract accessory glands - primarily the acid gland which, in the Apocrita, is the source of stinging venom - and arthrospores of the white-rot fungal pathogen, *Amylostereum areolatum*. Woodwasp larvae begin their lives tunneling through fungus-infested wood (14), ingesting wood fragments, fungal mycelia, and their own frass recolonized by *Amylostereum* (15,16). Larvae hatch from eggs deposited in a subset of the chambers drilled during oviposition. Larvae feed and grow inside the tree host for a one- to two-year period before emerging as adults. Before chewing their way out of the host tree following metamorphosis, female woodwasps sequester a new fungal inoculum in specialized organs called mycangia, where fungal spores are embedded in a waxy matrix. In addition to *A. areolatum*, *S. noctilio* is often associated with a parasitic nematode, *Deladenus siricidicola*, which can infect the reproductive tissues of male and female wasps (17). Because this nematode can greatly reduce the fertility of infected wasps, it has been harnessed as a biocontrol agent for controlling *S. noctilio* in commercial pine plantations (18,19).

Studies have shown that the woodwasp venom and *A. areolatum* must both be injected into a host tree to cause mortality (20-22), which indicates that specific components of the insect venom play critical roles in this pathology. Early biochemical analyses of *S. noctilio* venom, which was also called “mucus” in older literature (22-27), led to its characterization as an acid mucopolysaccharide-protein complex. An absence of sulfate groups indicated that heparin or chondroitin sulfate were not components of the venom (28). However, these early studies also failed to find sialic acid, indicating that the complex carbohydrates we now recognize as mucins,

distinguished by their copious sialic acid content (29), are also not constituents of the venom. On the other hand, the protein fraction from this venom was shown to harbor several common enzyme activities, including amylase, esterase, phenoloxidase, and protease.

When feeding or ovipositing, herbivorous insects release a variety of compounds that can be detected by host plants as signals for elevating defense responses (28,30-36). For example, β -glucosidase regurgitated onto cabbage leaves by *Pieris brassicae* larvae induces production of plant volatiles that attract parasitoid wasp predators of the caterpillar (34). Similarly, oviposition by the symphytan sawfly, *Diprion pini*, induces systemic reduction of photosynthetic rates as well as production of parasitoid-attractive volatiles in *Pinus sylvestris* (37). At the same time, other agents in insect secretions serve to counteract plant defenses. Thus, glucose oxidase regurgitated onto tobacco leaves by *Helicoverpa zea* larvae reduces production of nicotine, an inducible plant defense compound (38). And in extreme cases, such as the interactions between gall wasps and their host plants, insects emit chemicals that precisely alter growth and development of plant tissues to create structures that benefit the insect (30). Despite the obvious importance of chemical communications for modulating such plant-insect interactions, our understanding of these systems remains rudimentary. However, recent studies using high-throughput techniques, such as transcriptomic and proteomic analyses of salivary gland products, have opened new doors to understanding the chemical cross-talk between plants and herbivorous insects (39-41).

By contrast, venoms produced in the reproductive tract accessory glands of aculeate Hymenoptera, such as wasps and stinging ants, have been studied in great detail with respect to their modes of action and impact on human health (42,43). A few studies have even examined the effects of aculeate venoms on plants (44). Many individual active components in these

venoms have been purified and characterized and their specific targets identified (45,46), and some have been adopted for therapeutic purposes (47,48). However, novel activities in insect venoms are still being discovered; for example, recent work has highlighted novel antimicrobial properties associated with peptides and other components in many of these venoms (49), and high-throughput techniques are speeding discovery of new active agents (42,50,51).

Information on the molecular factors and responses that underlie the pathology in trees attacked by *S. noctilio* is required for efforts to develop trees with increased resistance to this insect. This study provides the foundation for work to identify specific proteins and peptides produced by *S. noctilio* that affect host defense responses. We also present molecular evidence for additional organisms with significant roles in the pathosystem. Whole transcriptome shotgun sequencing (RNA-Seq) of *S. noctilio* venom gland tissues was used to generate a catalog of coding sequences including those for secreted protein and peptide components of the venom. Annotations retrieved for the assembled transcripts identified sequences that could encode the previously reported enzyme activities. In addition, a variety of other sequences having relevance for understanding the effect of *S. noctilio* venom on pine trees were identified. As the first genomic dataset released for a basal species of Hymenoptera, this transcriptome assembly should find wide utility among researchers interested in phylogenetic studies of this important order of insects.

4.2 Materials and Methods

4.2.1 RNA Preparation

Eight newly-emerged female *S. noctilio* woodwasps were euthanized by freezing at -20° C. Venom sacs and attached venom glands were resected intact from woodwasp abdomens, forward of the oviduct where mixing of insect and fungal tissue occurs (Fig. 4.1), and immediately transferred to ice-cold RNALater buffer (Qiagen, Valencia CA). Samples were shipped on ice from Syracuse, NY to Athens, GA within three days. RNALater was decanted, and RNA was extracted from the tissues using a protocol modified from Lorenz et al. (100). Briefly, pooled venom sacs and glands were crushed using mortar and pestle in a final volume of 20 ml RNA extraction buffer (2 % (w:v) CTAB, 2 % (w:v) polyvinyl pyrrolidone, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 g/l spermidine, and 2 % (v:v) β -mercaptoethanol). The solution was extracted twice using chloroform followed by centrifugation, retaining the aqueous layer after each centrifugation. Pooled aqueous layers were brought to 10 M LiCl and incubated overnight at -20° C to precipitate nucleic acid. Following centrifugation, the pellet was resuspended in 1 M NaCl, 0.5% SDS, 10 mM Tris-HCl, pH 8.0, and 1 mM sodium EDTA (SSTE) buffer. Extractions using phenol/chloroform, pH 8, (PC8) and neat chloroform were used to further purify the RNA, retaining the aqueous layer after each centrifugation step. RNA was precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.0 volumes of cold 95% ethanol, followed by incubation overnight at -20° C. After centrifugation and decanting of the supernatant, the RNA pellet was resuspended in nuclease-free water and treated with RNase-free DNase (TurboDNA Free, Ambion, Austin TX). RNA quality and quantity were assessed by $A_{260}:A_{280}$ analysis and a sample (50 μ g) was sent for RNA-Seq analysis.

4.2.2 RNA Sequencing and Analysis

The Illumina sequencing library was prepared by staff in the Georgia Genomics Facility (GGF) at the University of Georgia (<http://dna.uga.edu/>) according to standard protocols. Complementary DNA (cDNA) prepared using random-hexamer primers was sheared using acoustic force (Covaris Mdl. S1, Woburn, MA) to generate a sequencing library having an average length of ca. 300 bp. A single lane of paired-end (PE 100) reads was performed at the Beijing Genomics Institute (BGI, Shenzhen, China) using an Illumina HiSeq 2000 instrument. Preliminary adapter filtering of raw read data was carried out at the BGI using proprietary methods. Raw reads were deposited in the GenBank Short Read Archive under accession number SRP022976.

Sequence reads were assessed using FastQC (101), and the data were determined to be of very high quality. Given the absence of contaminating adapters or primers, and a mean read sequence quality (Phred) score >38, quality trimming was deemed unnecessary. *De novo* transcriptome assembly was accomplished using Trinity ver. R2012-06-08 (102) run using the following settings (`--seqType fq --JM 196G --min_contig_length 100 --CPU 6 --bfly CPU 6 --output trinity_Sirex`). In order to determine the relative expression level of transcripts, paired-end reads were aligned to the assembled transcript clusters using the Trinity script `run_RSEM_align_n_estimate.pl`, which utilizes both the Bowtie aligner ver. 0.12.8 (103) for read mapping, and RSEM (104) for transcript quantification based on the calculation of a normalized expression value, transcripts per million (TPM), determined for each transcript isoform. Transcripts were annotated by querying against the NCBI non-redundant (nr) database using BLASTx with an Expect (E)-value cut-off of 1×10^{-10} (105). Assemblies that failed to return hits from the BLASTx analysis were queried against the NCBI nr nucleotide database using BLASTn

with an E-value cut-off of 1×10^{-10} . BLAST2GO (106) was used to identify gene ontology (GO) terms. First, a subset of transcripts was generated by employing a 1% cut-off for the RSEM isopercentage values associated with each transcript isoform in order to reduce the incidence of both extremely minor isoforms and isoforms that may have been generated via assembly errors. This subset of 28,974 transcripts was queried against the NCBI_nr database using BLASTx with an E-value cut-off of 1×10^{-5} . BLAST2GO was run using BLAST4pipe and the resulting .dat file was used to extract GO terms and GO IDs. Coding sequences were screened using Signal P 4.1 software (107) to identify proteins and peptides that could be secreted from venom gland cells. Nucleotide sequences first were translated in the reading frame matching BLASTx annotation, then truncated to the first methionine residue preceding BLAST homology before input to SignalP; where no methionine preceded the aligned translation, sequences were trimmed to first downstream methionine. The assembled clusters and BLASTx annotation results are housed in a MySQL database with a web-based interface and can be accessed at <http://128.192.15.239:8080/ngmweb/>. For the analyses reported in this paper (Tables 4.1-4.7), sequences with E-value $>10^{-20}$, TPM <0.2 , isopercentage value < 1 , or those returning annotations as ribosomal proteins were excluded.

For phylogenetic analyses of *S. noctilio* phenoloxidase sequences, the coding sequences for laccase-like multicopper oxidase (LMCO) genes from sixteen representative taxa were aligned prior to tree reconstruction. The nucleotide sequences and their corresponding amino acid sequences were obtained from the NCBI GenBank database using BLAST (108). In all, 51 sequences were retrieved and compiled for tree reconstruction. The amino acid alignment for the complete data set was performed using MAFFT (109), and molecular phylogeny estimates were derived using RAXML on a 1054-character sequence alignment (110). For the amino acid

alignment, the JTT substitution model with gamma distribution was used. A LMCO gene from the wood-rotting fungus, *Pleurotus ostreatus*, was used as an out-group to root the phylogenetic tree. Clade support was evaluated using 100 bootstrap replicates.

4.2.3 Zymogram and RT-PCR Analyses

Venom proteins were prepared by macerating 10 venom sacs in 100 mM sodium acetate, pH 5.5, buffer followed by filtration through glass fiber filters to remove cell debris. Samples from the resulting extract were mixed 50:50 with SDS-PAGE sample buffer and subjected to gel electrophoresis without prior boiling. Venom polypeptides were separated by non-denaturing SDS-PAGE in 4-15% gradient gels (Bio-Rad, Hercules CA) using a Tris-tricine buffer system (111). Protein bands were sized by comparison against Precision Plus protein size standards (Bio-Rad, Hercules, CA). Zymogram staining for laccase activity used 1,8-diaminonaphthalene (DAN) (112). Counter-staining of the DAN oxidation product using Coomassie brilliant blue dye was performed for 30 min at room temperature as previously described (112).

4.2.4 Non-*Sirex* sequences

Primers DS1-F and DS1-R corresponding to a *Deladenus siricidicola* microsatellite sequence (113), as well as genomic DNA (gDNA) samples from *Deladenus proximus* were obtained from Dr. Isabel Leal (Canadian Forest Service). *Deladenus siricidicola* (Kamona strain) obtained from Dr. David Williams (USDA-APHIS) was also used to prepare gDNA (114). Briefly, nematodes were ground in RNA extraction buffer heated to 65° C (115) and incubated 15 min. The resulting extract was mixed with 24:1 chloroform/isoamyl alcohol followed by centrifugation. DNA was precipitated from the aqueous supernatant by addition of an equal volume of isopropyl alcohol. The pellet recovered after centrifugation was washed with 70% ethanol, and the DNA was resuspended in nuclease-free water. PCR was carried out in 50 µl

reaction volumes consisting of 38 μ l water, 5 μ l 10X buffer (100 mM Tris-HCl, pH 8.8, 35 mM MgCl₂, 250 mM KCl), 2 μ l 10 mM dNTP mix, 1 μ l Taq polymerase, 3 μ l 5 μ M primer pair mix, and 1 μ l gDNA template. Reactions were run in a GeneAmp 9700 thermal cycler (Applied Biosystems, Carlsbad CA) under the following conditions: 95° C for 3 min, followed by 40 cycles of 94° C for 30 s, 55° C for 30 s, and 72° C for 90 s, followed by a final incubation at 72° C for 10 min. Amplimers were separated on 2 % agarose gels in TAE buffer, and bands were visualized by incubating with ethidium bromide for 20 min. Amplimer size estimates were based on comparisons with a Quick-Load 50 bp DNA Ladder (New England BioLabs, Ipswich, MA).

Degenerate primers for a conserved region of the *Wolbachia Wsp A* gene (116) were used to probe *S. noctilio* venom tissue cDNAs using PCR. Similarly-prepared cDNAs from *Wolbachia*-infected male *Nasonia vitripennis* wasps (supplied by Sarah Bordenstein, Vanderbilt University, Nashville, TN) were used as positive controls. PCR reactions were carried out as described above, except that 10 μ l of cDNA template was used, adjusting the water volume to 30 μ l, and performing only 30 cycles of amplification.

4.3 Results

4.3.1 Sequence assembly and annotation

A single lane of Illumina sequencing yielded ca. 68.7 GB of data and a total of 2.61×10^8 reads. Based on the results from FastQC analysis demonstrating an absence of contaminating adapter sequences as well as a high Phred 33 mean quality score value of 38, read data were not filtered prior to assembly. Assembly of the *S. noctilio* venom tissue transcriptome was performed using a minimum cut-off length of 100 bp, rather than 300 bp, to facilitate identification of transcripts that could encode small bioactive peptides. The assembly itself comprised 68,887

clusters including, as expected, a large number of short transcripts (i.e. 26,703 clusters (39%) were shorter than 200 bases in length). Another 18,960 clusters (28%) were at least 1 kb long, and 619 clusters (1%) were >8 kb. The mean cluster size was 1058 bases, and the largest cluster was 16.8 kb. Overall GC content was 38.5%. Collapsing closely related transcript isoforms in the collection of 68,887 clusters identified a set of 53,613 unique gene products that constitute the venom gland transcriptome.

Transcript clusters (hereafter referred to as transcripts) were queried against the NCBI non-redundant protein database using BLASTx. Low Expect values, along with high percent identity and percent similarity were taken to indicate reliable annotations, especially for abundant transcripts where read depth was high. Of the 68,887 transcripts queried, 24,471 (35%) returned annotations with E-values $\leq 1 \times 10^{-10}$. A total of 44,751 transcripts that either failed to return an annotation via BLASTx or returned an annotation above the E-value cutoff were analyzed using BLASTn. Using the same E-value cutoff of $\leq 1 \times 10^{-10}$, tentative annotations were assigned for another 776 transcripts following BLASTn analysis.

4.3.2 Transcript expression levels

Of the 24,471 transcripts that returned BLASTx annotations, 19,442 represented at least 1% of the total isoforms identified. Consistent with expectations for a transcriptome from an active glandular tissue, many of these abundant transcripts were predicted to encode proteins and enzymes associated with protein translation and RNA metabolism, including elongation factors, RNA helicases, and ribosomal proteins. The 20 most highly-expressed transcripts that returned annotation hits, excluding ribosomal proteins, are listed in Table 4.1. Among the 100 most abundant transcripts were a number that appeared to encode enzymes involved in the metabolism of complex polysaccharides, for example, an α -(1, 3) fucosyltransferase, which is the 13th most

abundant annotated transcript listed in Table 4.1. Also among the top 200 most abundant transcripts were many that appeared to encode enzymes recognized as contributors to venom activity in other wasps, including lipases, acid phosphatases, and vigilin (52). The coding sequences of highly abundant transcripts were analyzed for potential secretion signals using SignalP software, and of those examined ca. 10% were predicted to encode secreted proteins.

Annotations were not recovered for the seven most highly-expressed transcripts and nine of the top 50 most abundant transcripts failed to return any annotation (Table 4.2). However, only 17 total transcripts of unknown function (TUFs) were found among the 100 most abundant transcripts. These TUFs are of particular interest for the possibility that they encode venom constituents unique to the *Pinus-S. noctilio* interaction. Six of the 25 most abundant TUFs were between 100-200 nucleotides in length.

Transcripts returning annotations for ribosomal components (267 transcripts in total) comprised fully half (50) of the top 100 most abundant transcripts. To correlate gene expression with gene ontology, the 65 most abundant ribosomal transcripts were queried for GO terms. Among these, 34 transcripts (52%) gave GO terms identifying the cellular component as the ribosome. Sixty transcripts (92%) were identified as structural components of the ribosome, and 54 transcripts (83%) were identified as functioning in translation. Sixty-four transcripts (98%) gave GO identification terms as ribosome, small ribosomal subunit, or large ribosomal subunit.

4.3.3 Transcripts correlating with previously reported enzyme activity

In earlier work, Wong and Crowden (28) reported detecting amylase, esterase, phenoloxidase, and protease activities in *S. noctilio* venom. Transcripts that appear to encode proteins corresponding to each of the reported activities were identified in the venom gland transcriptome.

4.3.3.1 Amylase

Three sequences encoding apparent amylases in the *S. noctilio* transcriptome shared >60% identity at the amino acid level with putative amylases from an ant, a parasitoid wasp, and a bumblebee (Table 4.3). Mapped read counts indicated that one of these transcripts was very highly expressed relative to other transcripts (133rd most abundant) in venom gland tissues at the time the woodwasps were harvested and appeared to contain a signal peptide targeting it for secretion.

4.3.3.2 Esterase

Esterase annotations were returned for 23 sequences with TPM > 1, six of which contained apparent secretion signals (Table 4.4). The predicted proteins represented at least three major classes of esterases. Present among the esterase sequences with secretion signals were transcripts encoding apparent palmitoyl-protein thioesterase (10.1 TPM) and esterase FE4 (21.3 TPM), a class of enzymes overexpressed to confer resistance to organophosphate insecticides in some insects (53,54).

4.3.3.3 Phenoloxidase

The single most abundant transcript that returned an annotation, and the eighth most abundant transcript overall (6,662.9 TPM), was predicted to encode a homolog of a mosquito laccase-like multicopper oxidase (LMCO) with which it shared more than 50% identity over a

span of 600 amino acids (Table 4.5). The 656-amino acid sequence harbored an apparent signal peptide sequence, indicating that the product is likely secreted, as well as all four of the conserved copper-binding domains that characterize LMCOs (55). An additional sequence sharing homology with LMCOs was identified in the venom gland transcriptome, but the encoded protein lacks one of the four canonical copper-binding sites that characterize LMCOs. Further, the relative expression level of this transcript was low (2.1 TPM), and the predicted protein lacks an obvious signal sequence. In addition to the apparent LMCO sequences, transcripts containing sequences homologous to three bee phenoloxidases were also observed, one of which contained an apparent signal for secretion (Table 4.5).

Zymogram analysis using 1,8-diaminonaphthalene staining of non-denaturing SDS-PAGE gels confirmed the presence of laccase activity in an aqueous extract of *S. noctilio* venom (Fig. 4.2). The enzyme activity migrated with an apparent molecular mass in excess of 250 kDa under these conditions, suggesting that the native enzyme may preferentially exist in a multimeric state that is at least tetrameric. The aqueous venom extract readily oxidized syringaldazine (data not shown), a widely utilized laccase substrate whose oxidized products are soluble (56,57).

Gene trees for the two *S. noctilio* sequences related to LMCOs were reconstructed using a maximum-likelihood approach from which both the best tree (data not shown) as well as the bootstrap tree (Fig. 4.3) showed similar branching patterns. On the basis of the clade distribution and gene annotations, the tree could be broadly divided into a distinct clade for the recognized LMCOs, which included the highly expressed *S. noctilio* venom gland sequence, and a separate clade containing genes with homology to LMCOs. Each of these two clades contained sequences

from divergent insect orders, including representatives of the Hymenoptera, Diptera, Lepidoptera, and Coleoptera.

4.3.3.4 Proteases and peptidase

Of the 71 transcripts that returned annotations corresponding to proteases or peptidases with TPM > 1, the 15 harboring apparent secretion signals encoded at least 7 classes of protease or peptidase (Table 4.6). Several apparent serine protease (106.6 TPM), lysosomal aspartic protease (36.5 TPM), carboxypeptidase D (26.4 TPM) and furin-like protease (5.8 TPM) transcripts were found among the most highly expressed sequences. All but one of these secretion signal-containing transcripts bear closest homology to proteases from other hymenopteran spp.

4.3.4 Transcripts encoding potentially plant-like peptides

Many bioactive peptides and proteins in venoms act through their ability to mimic endogenous proteins in the target organism (58). From this premise the *S. noctilio* assemblies were searched for sequences annotated as having strong similarity to plant genes (Table 4.7). Only one of the three sequences recovered in this analysis was highly expressed, and it was annotated as being most homologous to a hypothetical protein (XP_003614388.1) predicted in the *Medicago truncatula* genome (59). This specific *M. truncatula* sequence has homologs in many other plant genomes, but there are few clues available at present as to possible functions for the encoded products of these genes. Despite the high relative expression level for this sequence, the transcript does not encode a product containing an obvious signal sequence. Thus, it remains to be seen whether an active venom constituent is derived from this sequence.

4.3.5 Non-Sirex sequences

4.3.5.1 Evidence of *Deladenus*-derived sequences

BLASTn analysis identified one *S. noctilio* transcript as a close match to a *Deladenus siricidicola* ribosomal RNA (expect value 9×10^{-53}). *D. siricidicola* is a parasitic nematode that burrows into *S. noctilio* larvae and infects the gonads, often leading to sterility in mature females (17). If the sampled woodwasps were parasitized, it is possible that nematodes could have accompanied the dissected venom glands used for sequencing library production (gonads and venom tissue are contiguous in adult female woodwasps). To further probe this possibility, genomic DNA was isolated from four pools of whole *S. noctilio* female woodwasps (five insects per pool, 20 insects total) collected near Syracuse, NY during a sampling period different from that used to collect wasps for the venom gland transcriptome library. PCR analysis using primers for the *D. siricidicola* DS-1 microsatellite sequence yielded amplicon bands from all four samples that matched bands from a positive control (gDNA purified from *D. siricidicola*, Kamona strain) (Fig. 4.4).

4.3.5.2 Evidence of *Wolbachia*-like transcripts

BLASTn analysis identified numerous *S. noctilio* transcripts as close matches for sequences previously seen in the genomes of multiple species of *Wolbachia*, a genus of α -proteobacteria whose members commonly exist as endosymbionts of various *Arthropoda* species (60). On closer inspection, these sequences appeared to encode transposases of a type typically associated with repetitive elements. PCR was used to probe cDNA and gDNA samples from additional *S. noctilio* females for sequences encoding the *Wolbachia* surface protein (WSP A). None of these reactions recovered amplified *Wsp A* sequences, indicating that our field-collected woodwasps most likely did not harbor *Wolbachia* sp.

4.4 Discussion

The reproductive tract accessory glands of *S. noctilio* have followed a unique evolutionary trajectory leading them to produce a venom that hampers the ability of pine trees to resist infection by the fungal pathogen vectored by this woodwasp, which in turn promotes larval survival (22). We previously demonstrated in two different species of pine that application of *S. noctilio* venom led to significant changes in pine gene expression, including increased expression of a large number of defense-response genes (61). This analysis of the *S. noctilio* transcriptome sets the stage for identification of active factors in the venom that cause changes in pine gene expression after oviposition.

Initial evaluation of the data indicated that ribosomal sequences are well-represented in the venom gland transcriptome, with 50 of the top 100 most abundant transcripts identified as such. Correlating gene ontology terms with gene relative expression confirmed that transcripts for protein expression and translation are among the most abundant present in the transcriptome of this tissue. High levels of expression for components of the cellular translation machinery are to be expected for secretory tissues whose primary function is to produce secreted proteins for the woodwasp venom.

4.4.1 Specific products of the *S. noctilio* venom gland

Early biochemical characterizations of whole *S. noctilio* venom suggested that a non-sulfated acid mucopolysaccharide was a major component (27). Subsequent studies of fractionated venom identified several enzymatic activities, including amylase, esterase, phenoloxidase, and protease (28). Our transcriptome analysis clearly shows that the *S. noctilio* venom gland cells are expressing components of the protein translation and glycosylation machinery in sufficient abundance to facilitate bulk production of glycoproteins and

polysaccharides for the venom. For example, one highly expressed transcript encodes an apparent fucosyltransferase (Table 4.1) that may be involved in the biosynthesis of the complex polysaccharide that contributes to the high viscosity of raw venom.

4.4.1.1 Amylase

SignalP software identified an obvious secretion signal in the most abundant amylase-encoding sequence identified in the transcriptome. Given the abundance of this sequence, the signal peptide presence, and the strong amylase activity previously reported in *S. noctilio* venom, it seems likely that this product is indeed secreted. While amylases have previously been identified as secreted products in insect digestive tracts (62,63), the function of this enzyme in venom is somewhat obscure. Considering the unique biology of the *S. noctilio* system, however, it is a reasonable hypothesis that the enzyme in venom is meant to hydrolyze starch from ruptured pine cells, which could provide glucose and/or maltose to stimulate growth of the fungus. This in turn suggests that another early effect of venom should be to cause lysis of pine cells around the site of oviposition so that starch is accessible to the amylase. It is unclear from previous literature, however, whether such lysis occurs under the influence of venom alone, although several of the enzymes predicted by the transcriptome annotation are capable of facilitating cell lysis, i.e., phosphatases, esterases, lipases.

4.4.1.2 Esterase

The original observation of esterase activity in *S. noctilio* venom used α -naphthol acetate as the substrate in a non-specific assay (28). Our analyses showed that transcripts putatively encoding secreted esterases were abundant in woodwasp venom tissue. Some of the venom esterases certainly could contribute to breakdown of cell membranes and lysis of host cells for starch release, particularly given the presence of abundant lipase and phosphodiesterase

transcripts observed in the venom transcriptome (*data not shown*). A number of fungal pathogens of trees are known to specialize in metabolizing fatty acids and triglycerides from their hosts (64). If *A. areolatum* is a similar specialist, then the woodwasp venom esterases may also serve to facilitate fungal nutrition at the oviposition site.

4.4.1.3 Phenoloxidase

Of the five phenoloxidases predicted in the venom transcriptome, only one contained both a signal sequence and the four canonical copper binding sites characterizing LMCOs. As one of the most highly expressed transcripts in the *S. noctilio* venom gland, it seems likely that this LMCO plays an important role in this pathosystem. Genes encoding LMCOs are ubiquitous in insect genomes (65). However, laccase activity has not been widely reported as a component of hymenopteran venoms, although two recent reports link LMCOs with venoms from parasitoid wasps (50,66). Consequently, it was a surprise to find a LMCO sequence as the most abundant annotated venom tissue transcript. The metabolic investment the woodwasp makes in this enzyme suggests an importance for reproductive success of the insect. Phylogenetic analyses showed the *S. noctilio* enzyme to be most closely related to an *Anopheles gambiae* LMCO whose putative function is to oxidize potentially toxic compounds from ingested sources (67). A similar function has been ascribed to a laccase secreted into the saliva of the green rice leafhopper (68). Detoxification of phenolic compounds is a function ascribed to many other LMCOs from both insect and fungal sources (65,69,70). Although a wide variety of functions have been ascribed to laccases in other organisms (71,72), the most reasonable function for the *S. noctilio* venom laccase would seem to be detoxification of phenolic defense compounds produced by the host tree around the site of oviposition, thereby protecting either the woodwasp eggs or the fungal inoculum (73,74).

Our phylogenetic analysis of the *S. noctilio* venom LMCO sequence utilized all available full-length LMCO gene sequences from reference genomes of insect species in the Hymenoptera, Diptera, Lepidoptera, and Coleoptera orders. That the *S. noctilio* (Hymenoptera) sequence maps more closely in this analysis to LMCO sequences from two Dipterans (*Anopheles gambiae* and *Drosophila melanogaster*) seems at first glance a bit surprising. However, the Siricidae are very much basal to the Hymenoptera and are quite diverged from the suborder Apocrita, which was the source of all other hymenopteran LMCO sequences used in this study. Also, as a symphytan, *S. noctilio* shares with *D. melanogaster* a reliance on plant matter as the milieu in which to oviposit and hatch their larvae, a distinction not shared by hymenopterans in the Apocrita. It may be that this particular LMCO form was lost in the lineage leading to the Apocrita as an ancestor moved away from living directly within plant tissues.

4.4.1.4 Protease and peptidase

Wong and Crowden noted azocoll-degrading (protease) activity in *S. noctilio* venom (28), and we detected several classes of proteinase-encoding sequences in the venom gland transcriptome. Undoubtedly, many of these are involved with the processing and maturation of various venom components. Others may act directly to impede the host plant's defense response or hasten tissue senescence (75-77). However, substantial work remains to link specific *S. noctilio* venom proteases with responses in the host trees.

4.4.1.5 Plant-like peptides

Several well-studied venom toxins act by mimicking components of target cell signaling systems and disrupting their normal function (78-80). That the *S. noctilio* venom transcriptome contains several sequences whose annotations match most closely to plant sequences suggests the possibility of similar mimicry at work in this system, but the idea remains to be tested. There

are no examples outside the Sirex-Pinus system of hymenopteran venoms having bioactivity tailored to affect plants. Based on studies of venom action mechanisms in other systems, however, it would be surprising to find that insects have not exploited this avenue for modifying plant responses to attack.

4.4.1.6 Unannotated sequences

Abundant sequences that currently remain unannotated (Table 4.2) may prove to be the most interesting found in our study. By their sheer abundance we expect these sequences to contribute bulk components that provide important structural and functional characteristics to the venom. Among these we may find sequences encoding glycosylated polypeptides that Wong and Crowden characterized as acidic mucopolysaccharides (28), and which make the venom viscous and sticky. We also hope to find among the TUFs one that encodes the heat-stable peptide that causes needle wilt when it is translocated into the crown of pines attacked by *S. noctilio* (81).

4.4.2 Other sequences

Transcripts that were annotated as *Deladenus* sequences were unexpected in *S. noctilio* venom gland tissue, particularly because none of the insects used for this study were associated with USDA APHIS studies of biocontrol measures for *S. noctilio*, which employed *D. siricidicola* (Kamona strain). However, when *S. noctilio* woodwasps are infected, juvenile *D. siricidicola* have been found free-living in the ovary and oviduct, which are adjacent and contiguous to the venom gland (17). Verification in this study of *Deladenus* sequences in additional woodwasp samples collected independently provides additional evidence for the parasitism of *S. noctilio* by indigenous North American strain(s) of *Deladenus* (82,83). Morris et al. (84) have also recently reported on parasitism of *S. noctilio* in the northeastern U.S. by native

Deladenus species. It remains to be seen what impact indigenous strains of *Deladenus* will have on *S. noctilio* populations or on the efficacy of biocontrol measures.

Unlike the situation with the *Deladenus* sequences, annotations suggesting the presence of *Wolbachia*-related transcripts did not lead to the discovery of widespread infection of *S. noctilio* with this bacterium, although such infections have been detected in hymenopteran parasitoids (85-87) as well as other symphytans (88). *S. noctilio* tissues probed using PCR did not contain sequences (*Wsp A*) indicative of active *Wolbachia* infection, and it appears that the *S. noctilio* sequences in question most closely resembled repetitive motifs related to transposable elements.

4.5 Conclusions

Assembly of the *Sirex noctilio* venom gland transcriptome will benefit the hymenopteran research community on two levels. First, these data represents the first transcriptomic analysis of a symphytan tissue, and offers insight into transcript abundance and predicted protein composition of hymenopteran venom from outside the suborder Apocrita. This data set contributes additional diversity and complexity to the set of gene families recognized in the Hymenoptera. Several variously structured phylogenetic trees for the Hymenoptera emphasizing different morphological, molecular, and genomic characters are currently under debate (4, 89-95). Because *S. noctilio* represents a basal suborder of the Hymenoptera, the transcriptome data presented in this study should provide an important new resource that taxonomists can use to sharpen phylogenetic inferences for this clade of insects. Comparative genomic and venomomic analyses will benefit from sequence data derived from a basal suborder of Hymenoptera.

In addition, comparison of similar gene families across hymenopteran venoms will offer insight into altered functions over an evolutionary time span. As other researchers have shown, venom polypeptide composition and function may be expected to correlate with insect behaviors, even within species (96-98). The venom transcriptome we present here is a first look at putative polypeptides whose functions in woodwasp survival do not have clear homologs in the parasitoid and stinging Hymenoptera. *S. noctilio* venom is not used directly either for defense or for capturing prey, as woodwasps have a phytophagous life plan that involves neither parasitizing other animals nor direct defense against them. No hymenopteran of suborder Apocrita is known to exert plant physiological effects via secreted venom, yet *S. noctilio* venom has acute and long-term effects on pine tree physiology (22,25,81,99). Indeed, it appears likely that the abundant laccase in venom plays a direct role in the pathosystem, most likely to detoxify host plant secondary metabolites; however, further characterization will be required to identify the exact target of this laccase and its function in the disease process. *S. noctilio* venom is expected to prove a rich source of novel phyto-active molecules for future study.

Acknowledgments

This research was supported by USDA NIFA Graduate Fellowship (2012-67011-19694) awarded to JMB, as well as the Robert McAlpin Scholarship. Some equipment used for the project was procured through an IIR Award to JMB from the UGA Graduate School. The authors wish to thank Kelly Zylstra for providing *S. noctilio* woodwasps, David Williams for providing samples of *D. siricidicola*, and Vic Mastro for use of facilities at USDA-APHIS PPQ, Syracuse, NY. Thanks also to Sarah Bordenstein for samples of *Wolbachia*-infected *Nasonia*, Mark Brown

for use of his microscopy facilities, and David Rutland for translation of critical references from German and photography assistance.

1

Table 4.1: The twenty most abundant transcripts from *S. noctilio* venom gland tissue that returned annotations, excluding ribosomal proteins. Relative abundance expressed as number of transcripts per million (TPM).

Sirex DB ID no.	Predicted secretion signal	TPM	Isoform %	Accession no.	Annotation	% Identity	% Conserved	Expect value
comp9052_c0_seq1	yes	6662.9	100%	ref XP_314845.4	AGAP008731-PA , LMCO [<i>A. gambiae</i> str. PEST]	37.5%	53.2%	1.00E-131
comp11343_c0_seq1	--	2343.2	100%	ref XP_003398391.1	polyadenylate-binding protein 1-like isoform 1 [<i>B. terrestris</i>]	90.6%	91.9%	0
comp7127_c0_seq1	--	1947.1	80%	ref NP_001166227.1	elongation factor 1-alpha [<i>N. vitripennis</i>]	97.0%	99.1%	0
comp11322_c1_seq1	--	1799.4	100%	gb EFZ13809.1	hypothetical protein SINV_04326 [<i>S. invicta</i>]	91.9%	97.1%	1.00E-110
comp7123_c2_seq1	--	1600.9	100%	ref XP_003614388.1	hypothetical protein MTR_5g051050, partial [<i>M. truncatula</i>]	56.7%	63.0%	5.00E-91
comp11352_c0_seq1	--	1359.5	100%	gb EFZ14326.1	hypothetical protein SINV_15101 [<i>S. invicta</i>]	79.4%	80.4%	1.00E-101
comp3276_c0_seq1	--	1348.1	100%	gb EFZ21522.1	hypothetical protein SINV_16043 [<i>S. invicta</i>]	83.6%	88.5%	1.00E-139
comp3319_c0_seq1	--	1310.1	100%	ref XP_003698913.1	uncharacterized protein LOC100865369 isoform 1 [<i>A. florea</i>]	90.4%	92.0%	3.00E-69
comp5116_c0_seq1	--	1188.0	100%	gb EFZ19789.1	hypothetical protein SINV_09784 [<i>S. invicta</i>]	91.3%	97.1%	1.00E-179
comp9360_c0_seq1	--	1106.1	100%	ref XP_003707066.1	activating transcription factor of chaperone-like [<i>M. rotundata</i>]	50.8%	59.8%	1.00E-100
comp5186_c0_seq1	--	1051.1	100%	ref XP_001602460.2	elongation factor 2-like [<i>N. vitripennis</i>]	93.8%	95.3%	0
comp5124_c0_seq1	--	921.6	100%	ref XP_001604593.1	ATP-dependent RNA helicase p62-like [<i>N. vitripennis</i>]	84.2%	89.0%	0
comp9077_c0_seq1	--	889.9	100%	ref XP_003695572.1	alpha-(1,3)-fucosyltransferase C-like [<i>A. florea</i>]	48.8%	67.2%	1.00E-132
comp3254_c0_seq1	--	757.0	100%	gb EFN71375.1	hypothetical protein EAG_03557 [<i>C. floridanus</i>]	87.2%	93.6%	3.00E-33
comp11419_c0_seq1	--	676.5	100%	ref XP_003396620.1	ADP,ATP carrier protein 2-like [<i>B. terrestris</i>]	90.0%	94.7%	0
comp7110_c0_seq1	yes	671.2	100%	ref XP_001601772.2	chitooligosaccharidolytic beta-N-acetylglucosaminidase-like [<i>N. vitripennis</i>]	66.6%	79.1%	0
comp5405_c0_seq1	--	653.8	100%	ref XP_001606906.1	stress-associated endoplasmic reticulum protein 2-like [<i>N. vitripennis</i>]	92.2%	95.3%	7.00E-30
comp11406_c0_seq1	--	596.8	100%	gb EFZ11726.1	hypothetical protein SINV_08231 [<i>S. invicta</i>]	95.8%	98.5%	1.00E-180
comp7127_c0_seq2	--	497.4	20%	ref NP_001166227.1	elongation factor 1-alpha [<i>N. vitripennis</i>]	97.0%	99.1%	0
comp8460_c0_seq1	--	462.6	100%	ref XP_001121565.1	beta-galactosidase-like [<i>A. mellifera</i>]	57.1%	73.2%	1.00E-112

Table 4.2: The twenty most abundant transcripts from *S. noctilio* venom gland tissue that did not return annotations. In cases where the longest reading frame did not contain a methionine, the next longest was chosen. Relative abundance expressed as number of transcripts per million (TPM).

Sirex DB ID no.	Assembly length (nt)	Longest ORF (nt)	TPM	Isoform %
comp11322_c2_seq2	397	201	464599.9	85.9
comp11325_c0_seq1	413	171	139531.8	100
comp3255_c1_seq1	1748	309	112735.3	100
comp11322_c2_seq1	502	210	76119.4	14.1
comp5157_c0_seq1	1014	315	35348.5	100
comp11331_c0_seq1	391	300	13056.8	100
comp3261_c1_seq1	1037	147	8199.9	100
comp11335_c0_seq1	455	267	4756.6	100
comp3258_c0_seq1	2370	336	725.9	100
comp279705_c0_seq1	101	87	698.1	100
comp11327_c0_seq1	125	78	611.8	100
comp7149_c0_seq1	381	291	502.9	84.9
comp3266_c0_seq1	558	174	457.2	100
comp9146_c0_seq1	679	198	379.0	100
comp10802_c0_seq3	536	354	332.4	49.7
comp11322_c3_seq1	211	114	317.8	100
comp8929_c1_seq1	451	450	301.9	100
comp10976_c7_seq1	820	390	265.5	100
comp10910_c0_seq2	153	150	258.6	47.7
comp20463_c0_seq1	107	84	252.34	100

Table 4.3: Transcripts from *S. noctilio* venom gland tissue encoding apparent amylases. Relative abundance expressed as number of transcripts per million (TPM).

Sirex DB ID no.	Predicted secretion signal	TPM	Accession no.	Annotation	Query match (a.a.)	% Identity	% Conserved	Expect value
comp10913_c0_seq1	yes	139.9	ref XP_003494475.1	alpha-amylase-like [<i>B. impatiens</i>]	489	65.4%	78.5%	0
comp182245_c0_seq1	--	0.3	gb EFN65423.1	alpha-amylase 3 [<i>C. floridanus</i>]	219	62.6%	77.2%	3.00E-97
comp5674_c0_seq1	--	0.2	emb CAR82255.1	alpha-amylase [<i>C. congregata</i>]	362	66.3%	76.5%	1.00E-175

Table 4.4: Transcripts from *S. noctilio* venom gland tissue encoding apparent secreted esterases. Relative abundance expressed as number of transcripts per million (TPM).

Sirex DB ID no.	TPM	Accession no.	Annotation	Query match (a.a.)	% Identity	% Conserved	Expect value
comp5854_c0_seq1	21.3	gb EGI57452.1	esterase FE4 [<i>A. echinator</i>]	563	59.0%	76.4%	0
comp5410_c0_seq1	6.2	gb EFN85555.1	putative esterase CG3488 [<i>H. saltator</i>]	400	81.3%	92.8%	0
comp9950_c0_seq1	5.6	ref NP_001153389.1	palmitoyl-protein thioesterase 2 precursor [<i>N. vitripennis</i>]	288	73.6%	85.4%	1.00E-158
comp9950_c0_seq2	3.0	ref NP_001153389.1	palmitoyl-protein thioesterase 2 precursor [<i>N. vitripennis</i>]	288	73.6%	85.4%	1.00E-159
comp8341_c0_seq1	1.9	ref XP_003689689.1	ectonucleotide pyrophosphatase/phosphodiesterase family member 4-like [<i>A. florea</i>]	293	61.8%	77.8%	1.00E-165
comp8724_c0_seq1	1.5	ref XP_001603353.1	palmitoyl-protein thioesterase 1-like [<i>N. vitripennis</i>]	279	66.3%	80.3%	1.00E-126

Table 4.5: Transcripts from *S. noctilio* venom gland tissue encoding apparent phenoloxidases. Relative abundance expressed as number of transcripts per million (TPM).

Sirex DB ID no.	Predicted secretion signal	TPM	Accession no.	Annotation	Query match (a.a.)	% Identity	% Conserved	Expect value
comp9052_c0_seq1	yes	6662.9	ref[XP_314845.4]	AGAP008731-PA [<i>A. gambiae</i> str. PEST], LMCO	616	37.5%	53.2%	1.00E-131
comp8866_c0_seq1	--	2.1	ref[XP_003393534.1]	laccase 4-like [<i>B. terrestris</i>]	292	31.2%	44.5%	7.00E-33
comp5673_c0_seq1	--	2.6	ref[XP_003697839.1]	phenoloxidase subunit A3-like [<i>A. florea</i>]	672	83.3%	91.4%	0
comp10581_c1_seq2	--	2.0	ref[XP_003694462.1]	peroxidase-like [<i>A. florea</i>]	570	59.8%	73.9%	0
comp10581_c1_seq1	yes	1.7	ref[XP_003694462.1]	peroxidase-like [<i>A. florea</i>]	597	61.0%	74.4%	0

Table 4.6: Transcripts from *S. noctilio* venom gland tissue encoding apparent secreted proteases or peptidases. Relative abundance expressed as number of transcripts per million (TPM).

Sirex DB ID no.	TPM	Accession no.	Annotation	Query match (a.a.)	% Identity	% Conserved	Expect value
comp8707_c1_seq1	56.6	gb EFN63907.1	serine protease snake [<i>C. floridanus</i>]	372	59.1%	75.5%	1.00E-156
comp14177_c0_seq1	36.5	ref XP_001600543.1	lysosomal aspartic protease-like [<i>N. vitripennis</i>]	368	76.6%	88.0%	0
comp9172_c0_seq1	31.7	gb EFN75742.1	serine protease easter [<i>H. saltator</i>]	365	60.5%	73.7%	1.00E-151
comp15972_c0_seq1	26.4	ref XP_003703306.1	carboxypeptidase D-like [<i>M. rotundata</i>]	421	71.7%	81.0%	0
comp3238_c0_seq1	14.4	ref XP_970438.1	similar to IMP1 inner mitochondrial membrane peptidase-like [<i>T. castaneum</i>]	146	62.3%	84.2%	4.00E-65
comp9350_c0_seq1	11.5	gb EGI69093.1	serine protease persephone [<i>A. echinator</i>]	375	56.5%	72.8%	1.00E-149
comp7362_c0_seq1	3.6	ref XP_003701890.1	membrane-bound transcription factor site-1 protease [<i>M. rotundata</i>]	1107	72.5%	81.6%	0
comp10988_c11_seq8	2.3	gb EGI69195.1	serine proteinase stubble [<i>A. echinator</i>]	339	72.6%	81.7%	1.00E-170
comp10988_c11_seq18	2.3	gb EGI69195.1	serine proteinase stubble [<i>A. echinator</i>]	339	72.6%	81.7%	1.00E-177
comp10664_c0_seq10	2.3	ref XP_003697527.1	furin-like protease 1, isoforms 1/1-X/2-like [<i>A. florea</i>]	578	83.6%	88.9%	0
comp5728_c0_seq1	2.3	gb EFN81067.1	uncharacterized peptidase C1-like protein F26E4.3 [<i>H. saltator</i>]	300	73.0%	84.3%	0
comp10988_c11_seq12	2.2	gb EGI69195.1	serine proteinase stubble [<i>A. echinator</i>]	339	72.6%	81.7%	1.00E-170
comp10664_c0_seq8	2.1	ref XP_003697527.1	furin-like protease 1, isoforms 1/1-X/2-like [<i>A. florea</i>]	578	83.6%	88.9%	0
comp10888_c1_seq1	1.4	ref XP_003398909.1	furin-like protease 2-like [<i>B. terrestris</i>]	1292	83.5%	89.5%	0
comp11243_c0_seq2	1.3	ref XP_003397354.1	disintegrin domain-containing protein 10-like [<i>B. terrestris</i>]	1040	64.3%	72.6%	0

Table 4.7: Transcripts from *S. noctilio* venom gland tissue returning annotations matching plant proteins. Relative abundance expressed as number of transcripts per million (TPM).

Sirex DB ID no.	TPM	Accession no.	Annotation	Query match (a.a.)	% Identity	% Conserved	Expect value
comp7123_c2_seq1	1600.9	ref XP_003614388.1	hypothetical protein MTR_5g051050, partial [<i>M. truncatula</i>]	300	56.7%	63.0%	5.00E-91
comp6949_c0_seq1	1.0	emb CAN61340.1	hypothetical protein VITISV_007301 [<i>V. vinifera</i>]	124	50.0%	70.2%	1.00E-47
comp6949_c0_seq3	1.9	emb CAN61340.1	hypothetical protein VITISV_007301 [<i>V. vinifera</i>]	91	54.9%	71.4%	9.00E-41

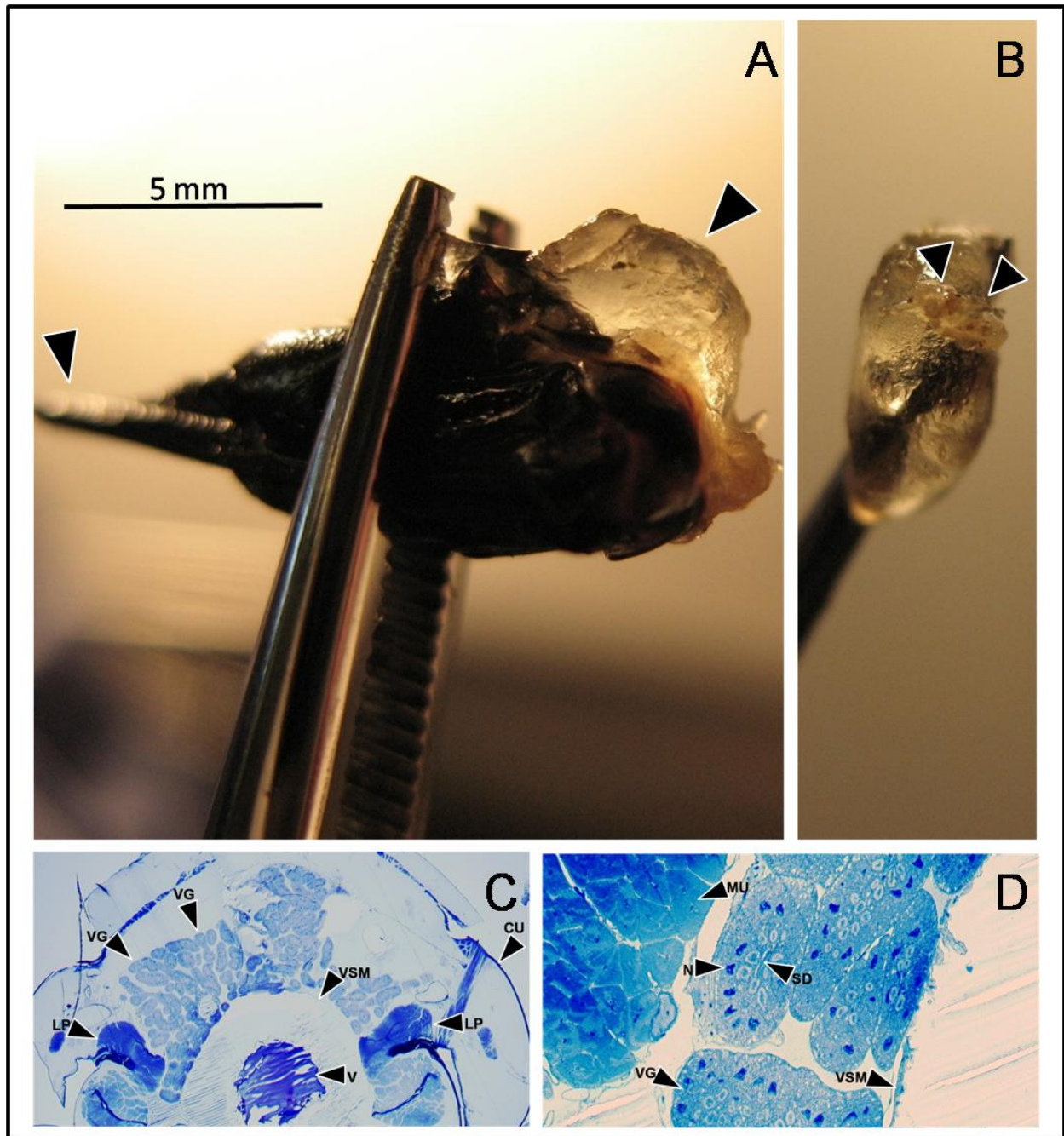


Figure 4.1: *Sirex noctilio* venom apparatus. Panel A, forceps holding the abdomen removed from an *S. noctilio* female. The venom sac (transparent globule) protrudes to the right (pointer) while the ovipositor extends out of focus to the left (pointer). Panel B, resected venom sac oriented to display the venom glands (small opaque projections, right upper third pointers). Panel C, posterior abdominal cross-section from a female *S. noctilio* stained with toluidine blue, 40 X. Panel D, same section, 400 X. Specific tissues and organs labeled as follows: CU, cuticle; LP, lateral pouch muscle attachment; MU, muscle; N, nucleus; SD, secretory duct of venom gland; V, venom contents; VG, venom gland; VSM, venom sac membrane (shrunken due to imperfect plastic impregnation).

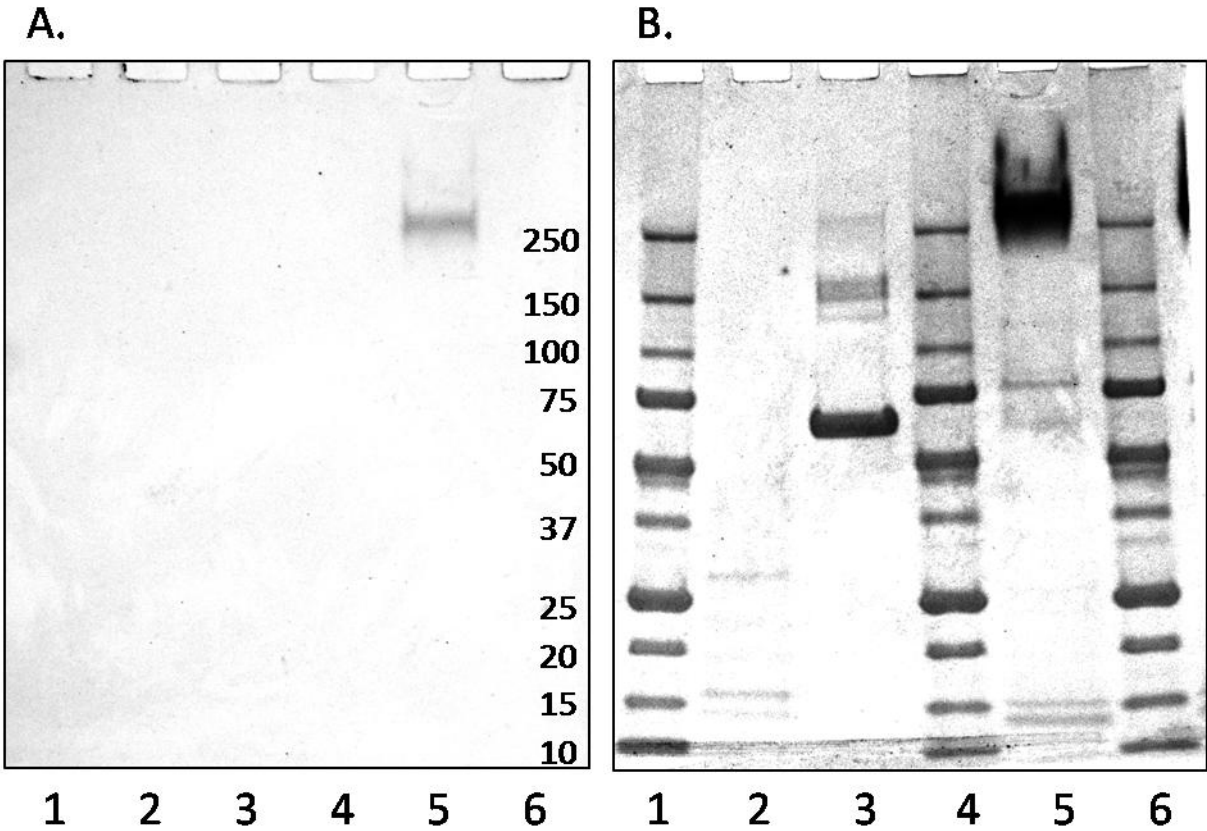


Figure 4.2: Zymogram analysis of laccase activity in aqueous extracts of *S. noctilio* venom glands. Panel A. Proteins resolved by non-denaturing SDS-PAGE in a 4-15% polyacrylamide gel were stained for laccase activity using 1,8-diaminonaphthalene (DAN) (42). Panel B. The same gel shown in Panel A following counterstaining for protein using Coomassie brilliant blue. Lane 1, protein size standards; lane 2, horseradish peroxidase; lane 3, bovine serum albumin (BSA); lane 4, protein size standards; lane 5, *S. noctilio* venom solution; lane 6, protein size standards.

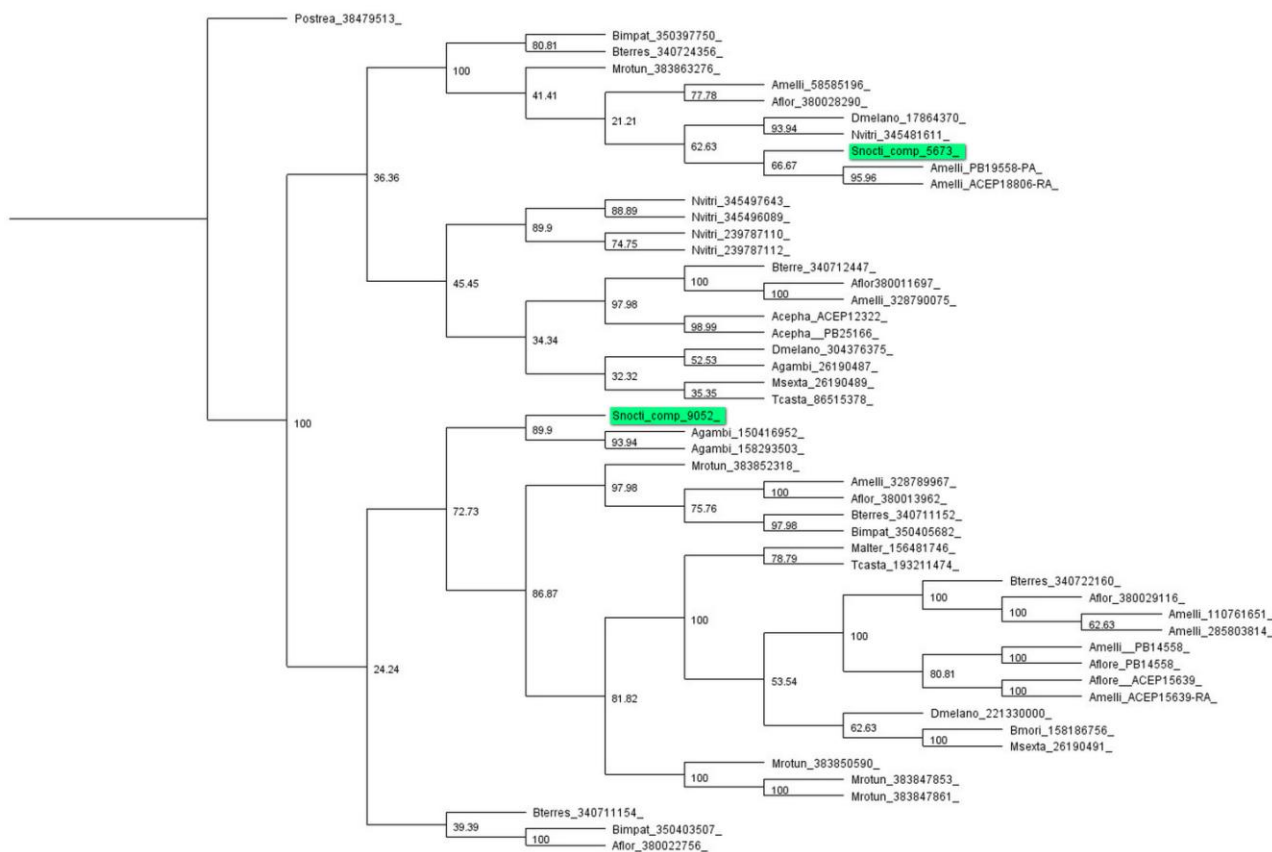


Figure 4.3: Laccase bootstrapping tree for the two principal putative phenoloxidase transcripts in *Sirex noctilio* venom tissue. The most abundant (*Sirex* DB ID no. comp9052_c0_seq1, read count 1,365,384) groups more closely to *Anopheles gambiae* than to any other hymenopteran laccase, It contains four putative conserved copper binding sites, a signal peptide sequence, and correlates with demonstrated laccase activity via zymogram analysis. The second phenoloxidase transcript (*Sirex* DB ID no. comp5673_c0_seq1, read count 582). This transcript contains only three putative copper binding sites, lacks a signal peptide (and thus was excluded from Table 4.5) sequence, and most likely serves an endogenous function for the insect.

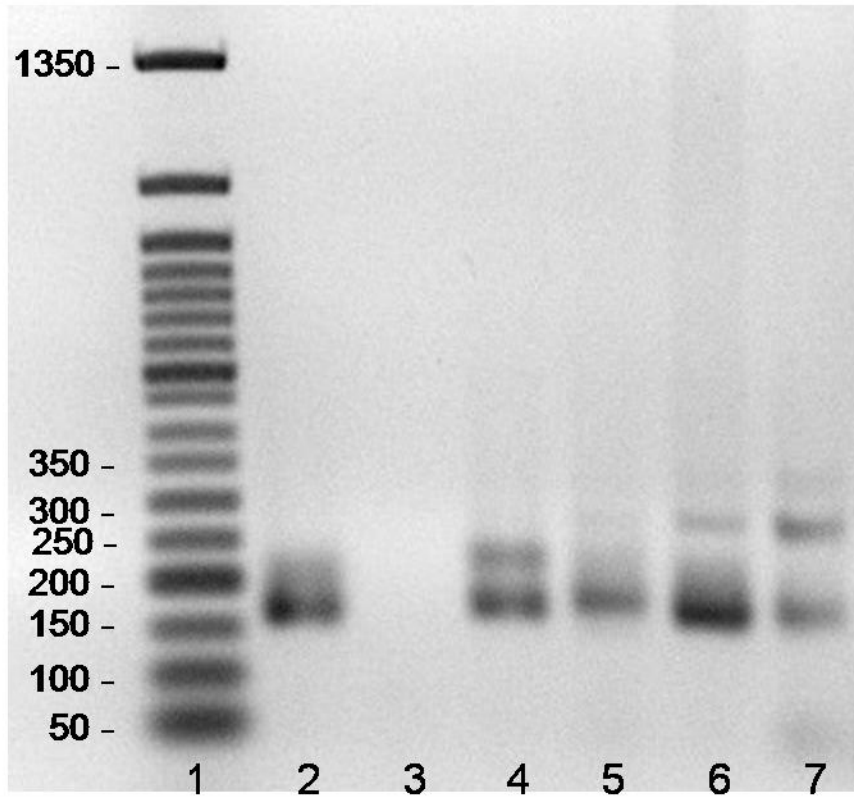


Figure 4.4: PCR test for presence of *Deladenus* sp. in *S. noctilio* females. *Deladenus* microsatellite primer DS-1 was used to probe four pools of gDNA prepared from pooled *S. noctilio* females. Lane 1, low MW DNA standard; lane 2, *D. siricidicola* gDNA (23 ng template); lane 3, negative control (no template); lanes 4-7, *S. noctilio* gDNA pools 1-4 (91, 140, 143, and 74 ng template, respectively).

References

1. Aukema, J. E., Leung, B., Kovacs, K., Chivers, C., Britton, K. O., Englin, J., Frankel, S. J., Haight, R. G., Holmes, T. P., Liebhold, A. M., McCullough, D. G., and Von Holle, B. (2011) Economic impacts of non-native forest insects in the continental United States. *PLoS One*
2. de Groot, P., Nystrom, K. L., and Scarr, T. A. (2006) Discovery of *Sirex noctilio* (Hymenoptera: Siricidae) in Ontario, Canada. *Great Lakes Entom.* **39**, 50-53
3. Hoebeke, E. R., Haugen, D. A., and Haack, R. A. (2005) *Sirex noctilio*: discovery of a palearctic siricid woodwasp in New York. *Newsl. Mich. Entomolo. Soc., Michigan*
4. Peters, R. S., Meyer, B., Krogmann, L., Borner, J., Meusemann, K., Schuette, K., Niehuis, O., and Misof, B. (2011) The taming of an impossible child: a standardized all-in approach to the phylogeny of Hymenoptera using public database sequences. *BMC Biol.* **9**, 2-14
5. Nygaard, S., Zhang, G., Schiøtt, M., Li, C., Wurm, Y., Hu, H., Zhou, J., Ji, L., Qiu, F., Rasmussen, M., Pan, H., Hauser, F., Krogh, A., Grimmelikhuijzen, C. J. P., Wang, J., and Boomsma, J. J. (2011) The genome of the leaf-cutting ant *Acromyrmex echinatior* suggests key adaptations to advanced social life and fungus farming. *Genome Res.* **21**, 1339-1348
6. Suen, G., Teiling, C., Li, L., Holt, C., Abouheif, E., Bornberg-Bauer, E., Bouffard, P., Caldera, E. J., Cash, E., Cavanaugh, A., Denas, O., Elhaik, E., Favé, M.-J., Gadau, J., Gibson, J. D., Graur, D., Grubbs, K. J., Hagen, D. E., Harkins, T. T., Helmkampf, M., Hu, H., Johnson, B. R., Kim, J., Marsh, S. E., Moeller, J. A., Muñoz-Torres, M. C., Murphy, M. C., Naughton, M. C., Nigam, S., Overson, R., Rajakumar, R., Reese, J. T.,

- Scott, J. J., Smith, C. R., Tao, S., Tsutsui, N. D., Viljakainen, L., Wissler, L., Yandell, M. D., Zimmer, F., Taylor, J., Slater, S. C., Clifton, S. W., Warren, W. C., Elsik, C. G., Smith, C. D., Weinstock, G. M., Gerardo, N. M., and Currie, C. R. (2011) The genome sequence of the leaf-cutter ant *Atta cephalotes* reveals insights into its obligate symbiotic lifestyle. *PLOS Gen.* e1002007
7. Bonasio, R., Zhang, G., Ye, C., Mutti, N. S., Fang, X., Qin, N., Donahue, G., Yang, P., Li, Q., Li, C., Zhang, P., Huang, Z., Berger, S. L., Reinberg, D., Wang, J., and Liebig, J. (2010) Genomic comparison of the ants *Camponotus floridanus* and *Harpegnathos saltator*. *Science* **329**, 1068-1071
 8. Smith, C. D., Zimin, A., Holt, C., Abouheif, E., Benton, R., Cash, E., Croset, V., Currie, C. R., Elhaik, E., Elsik, C. G., Fave, M.-J., Fernandes, V., Gadau, J., Gibson, J. D., Graur, D., Grubbs, K. J., Hagen, D. E., Helmkampf, M., Holley, J.-A., Hu, H., Viniegra, A. S. I., Johnson, B. R., Johnson, R. M., Khila, A., Kim, J. W., Laird, J., Mathis, K. A., Moeller, J. A., Muñoz-Torres, M. C., Murphy, M. C., Nakamura, R., Nigam, S., Overson, R. P., Placek, J. E., Rajakumar, R., Reese, J. T., Robertson, H. M., Smith, C. R., Suarez, A. V., Suen, G., Suhr, E. L., Tao, S., Torres, C. W., van Wilgenburg, E., Viljakainen, L., Walden, K. K. O., Wild, A. L., Yandell, M., Yorke, J. A., and Tsutsui, N. D. (2011) Draft genome of the globally widespread and invasive Argentine ant (*Linepithema humile*). *Proc. Natl. Acad. Sci. USA*, 5673-5678
 9. Smith, C. R., Smith, C. D., Robertson, H. M., Helmkampf, M., Zimin, A., Yandell, M., Holt, C., Hu, H., Abouheif, E., Benton, R., Cash, E., Croset, V., Currie, C. R., Elhaik, E., Elsik, C. G., Favé, M.-J., Fernandes, V., Gibson, J. D., Graur, D., Gronenberg, W., Grubbs, K. J., Hagen, D. E., Viniegra, A. S. I., Johnson, B. R., Johnson, R. M., Khila, A.,

- Kim, J. W., Mathis, K. A., Munoz-Torres, M. C., Murphy, M. C., Mustard, J. A., Nakamura, R., Niehuis, O., Nigam, S., Overson, R. P., Placek, J. E., Rajakumar, R., Reese, J. T., Suen, G., Tao, S., Torres, C. W., Tsutsui, N. D., Viljakainen, L., Wolschin, F., and Gadau, J. (2011) Draft genome of the red harvester ant *Pogonomyrmex barbatus*. *Proc. Natl. Acad. Sci. USA*, 5667-5672
10. Wurm, Y., Wang, J., Riba-Grognuz, O., Corona, M., Nygaard, S., Hunt, B. G., Ingram, K. K., Falquet, L., Nipitwattanaphon, M., Gotzek, D., Dijkstra, M. B., Oettler, J., Comtesse, F., Shih, C. J., Wu, W. J., Yang, C. C., Thomas, J., Beaudoin, E., Pradervand, S., Flegel, V., Cook, E. D., Fabbretti, R., Stockinger, H., Long, L., Farmerie, W. G., Oakey, J., Boomsma, J. J., Pamilo, P., Yi, S. V., Heinze, J., Goodisman, M. A. D., Farinelli, L., Harshman, K., Hulo, N., Cerutti, L., Xenarios, I., Shoemaker, D., and Keller, L. (2011) The genome of the fire ant *Solenopsis invicta*. *Proc. Natl. Acad. Sci. USA* **108**, 5679-5684
11. Werren, J. H., Richards, S., Desjardins, C. A., Niehuis, O., Gadau, J., Colbourne, J. K., and Nasonia Genome Working Group. (2010) Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. *Science* **327**, 343-348
12. Honeybee Genome Sequencing Consortium. (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* **443**, 931-949
13. Ryan, K., and Hurley, B. P. (2012) Life history and biology of *Sirex noctilio*. in *Sirex woodwasp and its fungal symbiont: research and management of a worldwide invasive pest* (Slippers, B., DeGroot, P., and Wingfield, M. J. eds.), Springer, New York. pp 15-30
14. Coutts, M. P., and Dolezal, J. E. (1965) *Sirex noctilio* and the physiology of *Pinus radiata*. 38th A.N.Z.A.A.S. Congress, Hobart, Australia

15. Francke-Grosmann, H. (1939) Über das Zusammenleben von Holzwespen (Siricinae) mit Pilzen. *Z. Angew. Entomol.* **25**, 647-680
16. Buchner, P. (1965) *Endosymbiosis of Animals with Plant Microorganisms*, J. Wiley & Sons, New York
17. Bedding, R. A. (1972) Biology of *Deladenus siricidicola* (Neotylenchidae), an entomophagous-mycetophagous nematode parasitic in siricid woodwasps. *Nematologica* **18**, 482-493
18. Bedding, R. A., and Akhurst, R. J. (1974) Use of the nematode *Deladenus siricidicola* in the biological control of *Sirex noctilio* in Australia. *J. Aust. Entomol. Soc.* **13**, 129-135
19. Iede, E. T., Penteadó, S. R. C., and Filho, W. R. (2012) The woodwasp *Sirex noctilio* in Brazil: monitoring and control. in *Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest* (Slippers, B., DeGroot, P., and Wingfield, M. J. eds.), Springer, New York. pp 217-228
20. Coutts, M. P. (1965) *Sirex noctilio* and the physiology of *Pinus radiata*: some studies of the interactions between the insect, the fungus, and the tree in Tasmania. in *Forestry and Timber Bureau* (D.N.D. ed., Forest Research Institute, Canberra, Australia
21. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .I. Effects of symbiotic fungus *Amylostereum* sp. (Thelophoraceae). *Aust. J. Biol. Sci.* **22**, 915-924
22. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .2. Effects of *S. noctilio* mucus. *Aust. J. Biol. Sci.* **22**, 1153-1161

23. Kile, G. A., Bowling, P. J., Dolezal, J. E., and Bird, T. (1974) The reaction of *Pinus radiata* twigs to the mucus of *Sirex noctilio* in relation to resistance to *Sirex* attack. *Aust. For. Res.* **6**, 25-34
24. Bowling, J. P., and Dolezal, J. E. (1970) Initial note on the development of a mucus test to determine resistance of *Pinus radiata* to attack by *Sirex noctilio*. *Aust. For. Res.* **5**, 57-62
25. Spradbery, J. P. (1973) A comparative study of the phytotoxic effects of siricid woodwasps on conifers. *Ann. Appl. Biol.* **75**, 309-320
26. Fong, L. K., and Crowden, R. K. (1973) Physiological effects of mucus from wood wasp, *Sirex noctilio* F., on foliage of *Pinus radiata* D. Don. *Aust. J. Biol. Sci.* **26**, 365-378
27. Boros, C. B. (1968) *The relationship between the woodwasp Sirex noctilio F. and the wood-rot fungus Amylostereum sp.* Master of Agricultural Science M. S., University of Adelaide
28. Wong, L. K., and Crowden, R. K. (1976) Preliminary studies on mucus secretion of wood wasp, *Sirex noctilio* F. 1. Physicochemical and biochemical properties. *Aust. J. Biol. Sci.* **29**, 21-32
29. Miner-Williams, W., Moughan, P. J., and Fuller, M. F. (2009) Methods for mucin analysis: a comparative study. *J. Agric. Food Chem.* **57**, 6029-6035
30. Hilker, M., and Meiners, T. (2011) Plants and insect eggs: how do they affect each other? *Phytochemistry* **72**, 1612-1623
31. Doss, R. P., Oliver, J. E., Proebsting, W. M., Potter, S. W., Kuy, S. R., Clement, S. L., Williamson, R. T., Carney, J. R., and DeVilbiss, E. D. (2000) Bruchins: insect-derived

- plant regulators that stimulate neoplasm formation. *Proc. Natl. Acad. Sci. USA* **97**, 6218-6223
32. Felton, G. W. (2008) Caterpillar secretions and induced plant responses. in *Induced Plant Resistance to Herbivory* (Schaller, A. ed.), Springer, Dordrecht. pp 369-387
 33. Bonaventure, G. (2012) Perception of insect feeding by plants. *Plant Biol.* **14**, 872-880
 34. Mattiacci, L., Dicke, M., and Posthumus, M. A. (1995) Beta glucosidase - an elicitor of herbivore-induced plant odor that attracts host -searching parasitic wasps. *Proc. Natl. Acad. Sci. USA* **92**, 2036-2040
 35. Afshar, K., Dube, F. F., Najafabadi, H. S., Bonneil, E., Thibault, P., Salavati, R., and Bede, J. C. (2013) Insights into the insect salivary gland proteome: diet-associated changes in caterpillar labial salivary proteins. *J. Insect Physiol.* **59**, 351-366
 36. Celorio-Mancera, M. D., Courtiade, J., Muck, A., Heckel, D. G., Musser, R. O., and Vogel, H. (2011) Sialome of a generalist lepidopteran herbivore: identification of transcripts and proteins from *Helicoverpa armigera* labial salivary glands. *PLoS One.* e26676
 37. Schröder, R., Forstreuter, M., and Hilker, M. (2005) A plant notices insect egg deposition and changes its rate of photosynthesis. *Plant Physiol.* **138**, 470-477
 38. Musser, R. O., Hum-Musser, S. M., Eichenseer, H., Peiffer, M., Ervin, G., Murphy, J. B., and Felton, G. W. (2002) Herbivory: caterpillar saliva beats plant defences - a new weapon emerges in the evolutionary arms race between plants and herbivores. *Nature* **416**, 599-600

39. DeLay, B., Mamidala, P., Wijeratne, A., Wijeratne, S., Mittapalli, O., Wang, J., and Lamp, W. (2012) Transcriptome analysis of the salivary glands of potato leafhopper, *Empoasca fabae*. *J. Insect Physiol.* **58**, 1626-1634
40. Su, Y.-L., Li, J.-M., Li, M., Luan, J.-B., Ye, X.-D., Wang, X.-W., and Liu, S.-S. (2012) Transcriptomic analysis of the salivary glands of an invasive whitefly. *PLoS One*. 10.1371/journal.pone.0039303
41. Francischetti, I. M. B., Lopes, A. H., Dias, F. A., Pham, V. M., and Ribeiro, J. M. C. (2007) An insight into the sialotranscriptome of the seed-feeding bug, *Oncopeltus fasciatus*. *Insect Biochem. Mol. Biol.* **37**, 903-910
42. de Graaf, D. C., Aerts, M., Danneels, E., and Devreese, B. (2009) Bee, wasp and ant venomics pave the way for a component-resolved diagnosis of sting allergy. *J. Proteomics* **72**, 145-154
43. Casewell, N. R., Wüster, W., Vonk, F. J., Harrison, R. A., and Fry, B. G. (2013) Complex cocktails: the evolutionary novelty of venoms. *Trends in Ecol. Evol.* **28**, 219-229
44. Metodiev, M. V. (1998) Hornet venom induces abundant expression of specific polypeptides in young barley leaves. *J. Exp. Bot.* **49**, 27-32
45. Hider, R. C. (1988) Honeybee venom: a rich source of pharmacologically active peptides. *Endeavour* **12**, 60-65
46. Lai, R., and Liu, C. (2011) Bioactive peptides and proteins from wasp venoms. in *Toxins and Hemostasis* (Kini, R. M., Clemetson, K. J., Markland, F. S., McLane, M. A., and Morita, T. eds.), Springer, Dordrecht. pp 83-95
47. Cavagnol, R. M. (1977) The pharmacological effects of Hymenoptera venoms. *Annu. Rev. Pharmacol. Toxicol.* **17**, 479-498

48. Monteiro, M. C., Romao, P. R. T., and Soares, A. M. (2009) Pharmacological perspectives of wasp venom. *Protein Pept. Lett.* **16**, 944-952
49. Moreau, S. J. M. (2013) "It stings a bit but it cleans well": venoms of Hymenoptera and their antimicrobial potential. *J. Insect Physiol.* **59**, 186-204
50. de Graaf, D. C., Aerts, M., Brunain, M., Desjardins, C. A., Jacobs, F. J., Werren, J. H., and Devreese, B. (2010) Insights into the venom composition of the ectoparasitoid wasp *Nasonia vitripennis* from bioinformatic and proteomic studies. *Insect Mol. Biol.* **19**, 11-26
51. Bouzid, W., Klopp, C., Verdenaud, M., Ducancel, F., and Vétillard, A. (2013) Profiling the venom gland transcriptome of *Tetramorium bicarinatum* (Hymenoptera: Formicidae): the first transcriptome analysis of an ant species. *Toxicon* **70**, 70-81
52. de Lima, P. R., and Brochetto-Braga, M. R. (2003) Hymenoptera venom review focusing on *Apis mellifera*. *J. Venom. Anim. Toxins* **9**, 149-162
53. Field, L. M., and Devonshire, A. L. (1998) Evidence that the E4 and FE4 esterase genes responsible for insecticide resistance in the aphid *Myzus persicae* (Sulzer) are part of a gene family. *Biochemical Journal* **330**, 169-173
54. Ono, M., Swanson, J. J., Field, L. M., Devonshire, A. L., and Siegfried, B. D. (1999) Amplification and methylation of an esterase gene associated with insecticide-resistance in greenbugs, *Schizaphis graminum* (Rondani) (Homoptera : Aphididae). *Insect Biochem. Mol. Biol.* **29**, 1065-1073
55. Gilbert, L. I. (2012) *Insect molecular biology and biochemistry*, 1st ed., CRC Academic Press, London

56. Harkin, J. M., Larsen, M. J., and Obst, J. R. (1974) Use of syringaldazine for detection of laccase in sporophores of wood rotting fungi. *Mycologia* **66**, 469-476
57. Harkin, J. M., and Obst, J. R. (1973) Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. *Experientia* **29**, 382-387
58. Higashijima, T., Uzu, S., Nakajima, T., and Ross, E. M. (1988) Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G-proteins). *J. Biol. Chem.* **263**, 6491-6494
59. Young, N. D., Debelle, F., Oldroyd, G. E. D., Geurts, R., Cannon, S. B., Udvardi, M. K., Benedito, V. A., Mayer, K. F. X., Gouzy, J., Schoof, H., Van de Peer, Y., Proost, S., Cook, D. R., Meyers, B. C., Spannagl, M., Cheung, F., De Mita, S., Krishnakumar, V., Gundlach, H., Zhou, S., Mudge, J., Bharti, A. K., Murray, J. D., Naoumkina, M. A., Rosen, B., Silverstein, K. A. T., Tang, H., Rombauts, S., Zhao, P. X., Zhou, P., Barbe, V., Bardou, P., Bechner, M., Bellec, A., Berger, A., Berges, H., Bidwell, S., Bisseling, T., Choisine, N., Couloux, A., Denny, R., Deshpande, S., Dai, X., Doyle, J. J., Dudez, A.-M., Farmer, A. D., Fouteau, S., Franken, C., Gibelin, C., Gish, J., Goldstein, S., Gonzalez, A. J., Green, P. J., Hallab, A., Hartog, M., Hua, A., Humphray, S. J., Jeong, D.-H., Jing, Y., Joecker, A., Kenton, S. M., Kim, D.-J., Klee, K., Lai, H., Lang, C., Lin, S., Macmil, S. L., Magdelenat, G., Matthews, L., McCarrison, J., Monaghan, E. L., Mun, J.-H., Najjar, F. Z., Nicholson, C., Noirot, C., O'Bleness, M., Paule, C. R., Poulain, J., Prion, F., Qin, B., Qu, C., Retzel, E. F., Riddle, C., Sallet, E., Samain, S., Samson, N., Sanders, I., Saurat, O., Scarpelli, C., Schiex, T., Segurens, B., Severin, A. J., Sherrier, D. J., Shi, R., Sims, S., Singer, S. R., Sinharoy, S., Sterck, L., Viollet, A., Wang, B.-B., Wang, K., Wang, M., Wang, X., Warfsmann, J., Weissenbach, J., White, D. D., White, J. D., Wiley, G. B.,

- Wincker, P., Xing, Y., Yang, L., Yao, Z., Ying, F., Zhai, J., Zhou, L., Zuber, A., Denarie, J., Dixon, R. A., May, G. D., Schwartz, D. C., Rogers, J., Quetier, F., Town, C. D., and Roe, B. A. (2011) The *Medicago* genome provides insight into the evolution of rhizobial symbioses. *Nature* **480**, 520-524
60. Werren, J. H., Baldo, L., and Clark, M. E. (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741-751
61. Bordeaux, J. M., Lorenz, W. W., and Dean, J. F. D. (2012) Biomarker genes highlight intraspecific and interspecific variations in the responses of *Pinus taeda* L. and *Pinus radiata* D. Don to *Sirex noctilio* F. acid gland secretions. *Tree Physiol.* **32**, 1302-1312
62. Franco, O. L., Rigden, D. J., Melo, F. R., and Grossi-de-Sa, M. F. (2002) Plant alpha-amylase inhibitors and their interaction with insect alpha-amylases: structure, function and potential for crop protection. *Eur. J. Biochem.* **269**, 397-412
63. Nation, J. L. (2008) *Insect Physiology and Biochemistry*, 2nd ed., CRC Press, Boca Raton, FL
64. Gao, Y., Breuil, C., and Chen, T. (1994) Utilization of triglycerides, fatty-acids and resin acids in lodgepole pine wood by a sapstaining fungus *Ophiostoma piceae*. *Mater. Organismen* **28**, 105-118
65. Dittmer, N. T., and Kanost, M. R. (2010) Insect multicopper oxidases: diversity, properties, and physiological roles. *Insect Biochem. Mol. Biol.* **40**, 179-188
66. Parkinson, N. M., Conyers, C. M., Keen, J. N., MacNicoll, A. D., Smith, I., and Weaver, R. J. (2003) cDNAs encoding large venom proteins from the parasitoid wasp *Pimpla hypochondriaca* identified by random sequence analysis. *Comp. Biochem. Physiol. C.Toxicol. Pharmacol.* **134**, 513-520

67. Lang, M. L., Kanost, M. R., and Gorman, M. J. (2012) Multicopper oxidase 3 is a laccase associated with the peritrophic matrix of *Anopheles gambiae*. *PLoS One*. e33985
68. Hattori, M., Konishi, H., Tamura, Y., Konno, K., and Sogawa, K. (2005) Laccase-type phenoloxidase in salivary glands and watery saliva of the green rice leafhopper, *Nephotettix cincticeps*. *J. Insect Physiol.* **51**, 1359-1365
69. Thurston, C. F. (1994) The structure and function of fungal laccases. *Microbiol.-SGM* **140**, 19-26
70. Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and Sannia, G. (2010) Laccases: a never-ending story. *Cell. Mol. Life Sci.* **67**, 369-385
71. Dean, J. F. D., and Eriksson, K. E. L. (1994) Laccase and the deposition of lignin in vascular plants. *Holzforschung* **48**, 21-33
72. Claus, H. (2004) Laccases: structure, reactions, distribution. *Micron* **35**, 93-96
73. Kessler, A., and Baldwin, I. T. (2002) Plant responses to insect herbivory: the emerging molecular analysis. *Annu. Rev. Plant. Biol.* **53**, 299-328
74. Dearing, M. D., Foley, W. J., and McLean, S. (2005) The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. *Annu. Rev. Ecol. Evol. Syst.* **36**, 169-189
75. Chen, H. J., Tsai, Y. J., Shen, C. Y., Tsai, T. N., Huang, G. J., and Lin, Y. H. (2013) Ectopic expression of sweet potato cysteine protease SPCP3 alters phenotypic traits and enhances drought stress sensitivity in transgenic *Arabidopsis* plants. *J. Plant Growth Reg.* **32**, 108-121
76. Roberts, I. N., Caputo, C., Criado, M. V., and Funk, C. (2012) Senescence-associated proteases in plants. *Physiol. Plant.* **145**, 130-139

77. Gomes, M. T. R., Oliva, M. L., Lopes, M. T. P., and Salas, C. E. (2011) Plant proteinases and inhibitors: an overview of biological function and pharmacological activity. *Curr. Protein Pept. Sci.* **12**, 417-436
78. Barber, C. M., Isbister, G. K., and Hodgson, W. C. (2013) Alpha neurotoxins. *Toxicon* **66**, 47-58
79. Shpakov, A. O. (2009) Polycationic peptides as nonhormonal regulators of chemosignal systems. *J. Evol. Biochem. Physiol.* **45**, 431-446
80. de Souza, B. M., da Silva, A. V. R., Resende, V. M. F., Arcuri, H. A., Cabrera, M. P. D., Neto, J. R., and Palma, M. S. (2009) Characterization of two novel polyfunctional mastoparan peptides from the venom of the social wasp *Polybia paulista*. *Peptides* **30**, 1387-1395
81. Bordeaux, J. M., and Dean, J. F. D. (2012) Susceptibility and response of pines to *Sirex noctilio*. in *Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest* (Slippers, B., DeGroot, P., and Wingfield, M. J. eds.), Springer, New York. pp 31-50
82. Yu, Q., de Groot, P., Leal, I., Davis, C., Ye, W., and Foord, B. (2009) Characterization of *Deladenus siricidicola* (Tylenchida: Neotylenchidae) associated with *Sirex noctilio* (Hymenoptera: Siricidae) in Canada. *Int. J. Nematol.* **19**, 23-32
83. Zylstra, K. E., and Mastro, V. C. (2012) Common mortality factors of woodwasp larvae in three northeastern United States host species. *J Insect Sci.* **12**, 1-8
84. Morris, E. E., Kepler, R. M., Long, S. J., Williams, D. W., and Hajek, A. E. (2013) Phylogenetic analysis of *Deladenus* nematodes parasitizing northeastern North American *Sirex* species. *J. Invertebr. Pathol.* **113**, 177-183

85. Kraaijeveld, K., Zwanenburg, B., Hubert, B., Vieira, C., de Pater, S., van Alphen, J. J. M., den Dunnen, J. T., and de Knijff, P. (2012) Transposon proliferation in an asexual parasitoid. *Mol. Ecol.* **21**, 3898-3906
86. Poorjavad, N., Goldansaz, S. H., Machtelinckx, T., Tirry, L., Stouthamer, R., and van Leeuwen, T. (2012) Iranian *Trichogramma*: ITS2 DNA characterization and natural *Wolbachia* infection. *Biocontrol* **57**, 361-374
87. Stouthamer, R., Breeuwer, J., Luck, R. F., and Werren, J. H. (1993) Molecular identification of microorganisms associated with parthenogenesis. *Nature* **361**, 66-68
88. Graham, R. I., Zahner, V., and Lucarotti, C. J. (2008) An intracellular symbiont and other microbiota associated with field-collected populations of sawflies (Hymenoptera : Symphyta). *Can. J. Microbiol.* **54**, 758-768
89. Sharkey, M. J. (2007) Phylogeny and classification of Hymenoptera. *Zootaxa*, 521-548
90. Rasnitsyn, A. P. (1979) *Origin and evolution of lower Hymenoptera*, Amerind, Washington, D.C.
91. Rasnitsyn, A. P. (1988) An outline of evolution of the hymenopterous insects (order Vespida). *Orient. Insects* **22**, 115-145
92. Vilhelmsen, L. (1997) The phylogeny of lower Hymenoptera (Insecta), with a summary of the early evolutionary history of the order. *J. Zoolog. Syst. Evol. Res.* **35**, 49-70
93. Carpenter, J. M., and Wheeler, W. C. (1999) Towards simultaneous analysis of morphological and molecular data in Hymenoptera. *Zoologica Scripta* **28**, 251-260
94. Zalut, S. M. (1997) Vespid venom analysis with phylogenetic inferences. *Biochem. Syst. Ecol.* **25**, 767-774

95. Dowton, M., and Austin, A. D. (1994) Molecular phylogeny of the insect order Hymenoptera - apocritan relationships. *Proc. Natl. Acad. Sci. USA* **91**, 9911-9915
96. Leluk, J., Schmidt, J., and Jones, D. (1989) Comparative studies on the protein composition of hymenopteran venom reservoirs. *Toxicon* **27**, 105-114
97. Schmidt, J. O. (1982) Biochemistry of insect venoms. *Annu. Rev. Entomol.* **27**, 339-368
98. Baracchi, D., and Turillazzi, S. (2010) Differences in venom and cuticular peptides in individuals of *Apis mellifera* (Hymenoptera: Apidae) determined by MALDI-TOF MS. *J. Insect Physiol.* **56**, 366-375
99. Coutts, M. P. (1968) Rapid physiological change in *Pinus radiata* following attack by *Sirex noctilio* and its associated fungus, *Amylostereum* sp. *Aust. J. Sci.* **30**, 275-277
100. Lorenz, W. W., Yu, Y.-S., and Dean, J. F. D. (2010) An improved method of RNA isolation from loblolly pine (*P. taeda* L.) and other conifer species. *J. Vis. Exp.* **36**
101. Andrews, S. (2013) FastQC: a quality control tool for high throughput sequence data. Babraham Institute, Cambridge, UK
102. Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., and Regev, A. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **29**, 644-U130
103. Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* R25
104. Li, B., and Dewey, C. (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323

105. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410
106. Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M., and Robles, M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674-3676
107. Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**, 785-786
108. Benson, D., Boguski, M., Lipman, D., Ostell, J., Ouellette, B., Rapp, B., and Wheeler, D. (1999) GenBank. *Nucl. Acids Res.* **27**, 12-17
109. Katoh, K., Misawa, K., Kuma, K.-I., and Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucl. Acids Res.* **30**, 3059-3066
110. Stamatakis, A., Ott, M., and Ludwig, T. (2005) RAxML-OMP: an efficient program for phylogenetic inference on SMPs. in *Parallel Computing Technologies* (Malyskin, V. ed.), Springer, Berlin. pp 288-302
111. Schägger, H., and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368-379
112. Hoopes, J. T., and Dean, J. F. D. (2001) Staining electrophoretic gels for laccase and peroxidase activity using 1,8-diaminonaphthalene. *Anal. Biochem.* **293**, 96-101
113. Leal, I., Foord, B., Davis, C., de Groot, P., Mlonyeni, X. O., and Slippers, B. (2012) Distinguishing isolates of *Deladenus siricidicola*, a biological control agent of *Sirex*

- noctilio*, from North America and the southern hemisphere using PCR-RFLP. *Can. J. For. Res.* **42**, 1173-1177
114. Zouache, K., Voronin, D., Tran-Van, V., Mousson, L., Failloux, A. B., and Mavingui, P. (2009) Persistent *Wolbachia* and cultivable bacteria infection in the reproductive and somatic tissues of the mosquito vector *Aedes albopictus*. *PLoS One*. e6388
115. Lorenz, W. W., Sun, F., Liang, C., Kolychev, D., Wang, H. M., Zhao, X., Cordonnier-Pratt, M. M., Pratt, L. H., and Dean, J. F. D. (2006) Water stress-responsive genes in loblolly pine (*Pinus taeda*) roots identified by analyses of expressed sequence tag libraries. *Tree Physiol.* **26**, 1-16
116. Jeyaprakash, A., Hoy, M. A., and Allsopp, M. H. (2009) Multiple *Wolbachia* strains in *Apis mellifera* Capensis from South Africa. *Apidologie* **40**, 178-183

CHAPTER 5

NOCTILISIN, A VENOM GLYCOPEPTIDE, IS THE HEAT-STABLE FACTOR THAT
CAUSES NEEDLE WILT AND DEFENSE GENE RESPONSES IN PINES ATTACKED BY
THE SIREX NOCTILIO WOODWASP⁴

⁴ Bordeaux, J.M., W.W. Lorenz, D. Johnson, M.J. Badgett, J. Glushka, R. Orlando, and J.F.D. Dean. Submitted to the Journal of Biological Chemistry, 02/21/2014.

Abstract

During oviposition, female *Sirex noctilio* woodwasps inject their conifer hosts with a venom gland secretion that induces a variety of physiological changes that facilitate subsequent lethal infection by a symbiotic fungus. A heat-stable factor that can migrate from the site of oviposition in the trunk through the xylem to needles in the crown of attacked pines was purified by size-fractionation and RP-HPLC using activity assays based on defense gene induction as well as the needle wilt response in pine shoot explants. An 11-amino acid, post-translationally modified peptide (SEGPROGTKRP) encoded by the most abundant transcript recovered from *S. noctilio* venom gland tissue comprised the backbone of the 1850 Dalton active factor. Post-translational modifications included hydroxylation of a Pro residue at position 6 as well as O-glycosylation of Ser and Thr residues at positions 1 and 8, respectively. The O-linked sugars were identical α -linked N-acetylgalactosamine residues modified at the C6 position by addition of phosphoethanolamine. In contrast to the native peptide, a synthetic version of the hydroxylated peptide backbone lacking the glycosyl side chains failed to induce pine defense genes or cause needle wilt in excised shoots. This peptide, hereafter called noctilisin, is related to the O-glycosylated short-chain proline-rich antimicrobial peptides exemplified by drosocin. The noctilisin structure suggests possibilities for blocking its defense response suppression activity in pines attacked by *S. noctilio* and should yield new insight into the surveillance systems plants use to monitor insect attack.

5.1 Introduction

Sirex noctilio (Fabricius), an Eurasian woodwasp of the horntail family, is a member of the basal suborder Symphyta within the Hymenoptera. In its native habitat, *S. noctilio* is not known for causing significant damage to its conifer hosts, but where it has been introduced in the southern hemisphere it has caused substantial economic losses in commercial pine plantations and is the target of costly and extensive biocontrol efforts (1). *S. noctilio* has recently become established in the region around Lake Ontario (2,3), and there is concern that this invasive pest could cause widespread damage in North American forests. Through study of the molecular mechanisms governing interactions between *S. noctilio*, its conifer hosts, and the symbiotic fungal pathogen vectored by the woodwasp we hope to identify ways to reduce tree mortality and increase our understanding of the systems employed by plants to recognize and react to insect pests.

S. noctilio attacks conifers as part of the reproductive process for these insects (4). Females use their ovipositors to bore through the cambium of host trees (conifers, especially *Pinus* spp.) and then inject secretions from reproductive tract accessory glands, predominantly venom from the acid glands, along with eggs and oidia of a symbiotic white-rot fungus, *Amylostereum areolatum*. A few days after oviposition, needles in the tree crown begin to bend or ‘flag’ at the fascicle sheath and then present wilting and yellowing symptoms described in the literature as resembling premature senescence (5). Other early responses seen in pines attacked by *S. noctilio* include increased stem respiration and decreased photosynthate transport from photosynthetic tissues. Profuse beading or streaming of resin is sometimes evident at sites of oviposition on heavily attacked trees. Because these early responses occur before egg hatching or significant fungus growth they have been generally attributed to the woodwasp venom, also

referred to as ‘mucus’ in the early literature describing this pathosystem (6). With the tree in a state of weakened defense from envenomation, the oidia spores of *A. areolatum* initiate mycelial growth and the fungus spreads, feeding off the surrounding wood. After the eggs hatch, *S. noctilio* larvae tunnel through the wood, ingesting fungal mycelia and fungus-altered wood. After 1-2 years and several molts, adult insects emerge to mate and start the cycle again. *S. noctilio* prefers to attack suppressed or weakened trees that often can be killed within a single season. However, even healthy dominant trees can be killed if they experience multiple attacks from the woodwasp, suggesting that progression of the pathology requires dose-dependent repression of pine defense responses.

Early studies of the *S. noctilio* interaction with pines identified several effects and responses that were associated with specific components of the pathosystem (6,7). One of the most notable of these was the wilting of needles when venom alone was applied to cut stems or bore holes in the tree trunk. A heat-stable, water-soluble fraction from *S. noctilio* venom was subsequently shown to induce the same responses (8,9). However, venom alone was not lethal to treated trees, and mortality was only seen when trees were inoculated with both the venom and the fungus (6).

Generally speaking, hymenopteran (apocritan) venoms are highly variable mixtures of bioactive polypeptides and small molecules (10-14); however, it was unclear to what extent this was true for the viscous *S. noctilio* venom described by early researchers as an acid mucopolysaccharide-protein complex (9,15). Xylem mobility of a factor causing needle wilt suggested a small, water-soluble molecule, and stability to boiling temperatures or autoclaving was taken as evidence against the factor being an enzyme or large polypeptide (9,16). However, sensitivity to protease digestion (17) suggested to us that the active factor might be a bioactive

peptide, particularly as many such molecules have been found in other hymenopteran venoms (18). Quantitative real-time PCR (qRT-PCR) assays that measured induction of pine pathogenesis-related protein (PR4) and thaumatin-like protein (TLP) gene expression in response to the heat-stable activity (17) were used to follow the wilt-inducing factor during venom fractionation. Purification and characterization of the active factor are described here.

5.2 Experimental Procedures

5.2.1 Purification and activity testing

Venom glands were extracted from *S. noctilio* females provided by Kelley Zylstra (USDA APHIS, Syracuse, NY). Live adult insects were frozen after emergence from pine logs and stored at -80° C until use. Frozen wasps were submerged briefly in ethanol and then dissected to remove the venom sac and glands. Pooled tissues were crushed in reagent-grade water using a Dounce homogenizer and the resulting suspension was diluted to a final concentration of 20 mg/ml based on whole tissue weight (Fraction 1). This fraction was boiled 15 min, cooled and dispensed into 1-ml aliquots prior to storage at -20° C. After thawing, the solution was centrifuged at 17,000 x g for 5-10 min, and the clarified supernatant was retained (Fraction 2). Using a centrifugal concentrator (Pall, Ann Arbor, MI USA), the supernatant was concentrated against a 10-kDa MWCO membrane at 6,700 x g, and the pass-through volume was retained (Fraction 3). Reversed-phase HPLC (RP-HPLC) separation of Fraction 3 was carried out using a Zorbax Eclipse Plus C18 column, 4.6 x 250 mm, 5 µm (Agilent, Santa Clara, CA USA). Chromatographic conditions were as follows: the flow rate was 1.2 ml/min, mobile phase A was 0.07% TFA in water, mobile phase B was 0.04% TFA in acetonitrile, and the elution

profile was 100% mobile phase A for 30 min, followed by a 0-10% linear gradient of mobile phase B over 60 min. Elution was monitored as needed at 210, 254, and 280 nm.

Eluent fractions collected from the RP-HPLC column were normalized according to an estimate of 50% loss-on-column; thus, material in each fraction from a 500- μ l injection was lyophilized (Eppendorf Vacufuge, Hamburg, Germany) and resuspended in 250 μ l of reagent-grade water. The activity contained in each fraction was measured by dosing individual shoot tips from sensitive *P. radiata* plants maintained in a growth chamber with a 50 μ l aliquot of the water suspension followed by 24-h incubation. Procedures for RNA extraction, cDNA synthesis, and qRT-PCR quantitation were described previously (17). Briefly, 5 μ l of 0.5 μ M primer pair, 5 μ l of 0.5 μ M cDNA template, and 10 μ l SybrGreen 2X Supermix (BioRad, Hercules, CA) were mixed, and the 20 μ l reactions were run on an iCycler thermal cycler (BioRad, Hercules, CA) using the following program: 95° C for 3 min, 40 cycles at 95° C for 30 s, 65° C for 45 s, and 78° C for 20 s, followed by 95° C for 1 min, 55° C for 1 min. Post-amplification melt-curve analysis used 81 cycles from 55° to 95° C for 10 s, incremented at 0.5° C. All qRT-PCR procedures utilized both PR4 and TLP as probes and expression levels were normalized to ACT1 (actin). Assay results were deemed acceptable when three replicate wells agreed at <0.5% RSD.

At various stages of the purification process, results from the qRT-PCR assays were validated against a needle-wilt bioassay. Briefly, 3-month old *P. radiata* seedlings were cut at the soil line and dosed with 50 μ L purified factor (97 μ M noctilisin), 20 mg/ml soluble whole venom, or water as a control. Activity was monitored over several days during which time visible wilting of seedling needles was sometimes accompanied by needle discoloration.

5.2.2 Molecular weight determination

The active factor recovered from RP-HPLC was subjected to mass spectrometry analysis using an AB SCIEX TOF/TOF 5800 System (Framingham, MA USA) by suspending 7.5 mM peptide (50x whole venom equivalent) in a matrix consisting of α -cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO USA) and a v/v 50/50/1 mixture of acetonitrile/water/formic acid. Samples were spotted both with and without internal standards, including human [Glu]-1-fibrinopeptide B (MW 1570.57 Da) and calcitonin gene-related peptide fragment (CGRP₈₋₃₇, MW 3125.59 Da). A separate aliquot of 7.5 mM concentrated peptide was subjected to C18 capillary separation (Halo Peptide ES-C18, 150 mm x 200 μ m, 5 μ m Advanced Materials Technology, Wilmington, DE USA) with electrospray ionization (ESI) mass spectrometry (Finnegan LTQ, Thermo Fisher Scientific, Waltham, MA USA). The M+2H⁺ fragment m/z 925 (peak corresponding to 1850 Da) was chosen for fragmentation via collision-induced dissociation (CID).

5.2.3 Sequence analysis

The bioactive material recovered after RP-HPLC was subjected to protein sequence analysis by Edman degradation at the Synthesis & Sequencing Facility at Johns Hopkins University. The purified peptide was subjected to a second round of sequencing after the N-terminus was deblocked using an acid and heat treatment (19).

The *S. noctilio* venom gland transcriptome characterized using Illumina DNA sequencing to generate more than 24,000 unique transcript assemblies was previously described (Bordeaux et al., submitted). The amino acid sequence identified by N-terminal sequencing corresponded to a short open reading frame (ORF) in the most abundant transcript of the venom gland transcriptome. To verify the nucleotide sequence predicted from the Illumina assembly, cDNA

prepared from venom gland tissue was probed with PCR primers designed using Primer3 software (20). The primers (comp11322_L: 5'-ACT CAG ATG TACT CG TGA AA-3'; comp11322_R: 5'-TGT CGT AAC ATC CGT ATA GA-3') were designed to amplify a 257-bp fragment that fully encompassed the predicted ORF. The resulting amplicon product was sequenced in the Georgia Genomics Facility at UGA.

5.2.4 Peptide synthesis

As total synthesis of mature noctilisin was impractical, a synthetic peptide backbone (non-glycosylated SEGPROGTKRP, 1197 Da) was prepared at the Synthesis & Sequencing Facility at Johns Hopkins University. The biomarker gene expression assays described previously were used to assess bioactivity of the non-glycosylated synthetic peptide (aglyconoctilisin) versus the glycopeptide purified from venom glands. In these assays, 50 μ l aliquots of 30 μ M and 300 μ M of synthetic peptide solutions in water were applied to excised shoot tips.

5.2.5 Molar absorptivity and dose-dependent response

Molar absorptivity of the venom peptide in reagent-grade water was determined by measuring absorbance at 190 nm using an Agilent 8453 diode array spectrophotometer (Agilent, Santa Clara, CA USA). Assuming a per-residue molar coefficient of 10,000/M x cm for amino acid residues, the 11-amino acid synthetic peptide was calculated to have $\epsilon_{190} = 110,000$ (21,22). This value was subsequently verified using aglyconoctilisin and presuming absorbance at this wavelength from the glycans is negligible.

Dose-dependence experiments were performed using the biomarker assays described above. Aliquots (50 μ L) of noctilisin serially-diluted in reagent-grade water were administered to shoot tips cut from two *P. radiata* trees that had been maintained in a growth chamber. Activity

was measured for purified noctilisin at concentrations ranging from 6.5 – 97 μM (0.33 – 4.9 nmoles applied to each shoot tip).

5.2.6 NMR structural characterization

Purified peptide (~90 nmoles) was dissolved in 80 μL D_2O to exchange all labile protons. Standard 2D COSY, TOCSY, ROESY and HSQC spectra were collected on an Agilent 600 MHz DD2 spectrometer (Agilent, Santa Clara, CA USA) equipped with a 3-mm cryogenic probe at 25 $^\circ\text{C}$. A 1D (^1H) ^{31}P HMQC experiment was collected using a Varian Inova 500 MHz spectrometer (Agilent, Santa Clara, CA USA) with an HX probe tuned to ^{31}P . The sample was then exchanged with H_2O and a 500 ms mixing time NOESY dataset was collected using a 900 MHz DD2 spectrometer. Data was processed using Mnova software (Mestrelab, Inc.).

5.3 Results

5.3.1 Purification and molecular weight determination

RP-HPLC separated the heat-treated venom gland Fraction 3 (10 kDa MWCO pass-through (Purification and Activity above) into numerous peaks of various sizes detected at 254 nm; activity was limited to the third and fourth fractions between 40 and 60 minutes (Figure 5.1). Monitoring at 280 nm revealed nearly all the activity was confined to a single poorly-absorbing peak at 60 min (Figure 5.2). Changing monitoring wavelength to 210 nm revealed a series of well-resolved symmetric peaks, and activity testing narrowed focus to a single large peak eluting from the column at about 60 min, corresponding to the low-absorbing peak at higher wavelengths (Figure 5.3).

MALDI-TOF/MS detected a single component (noctilisin) (Figure 5.4B) having an internal standard calibrated mass of 1849.95 Da (Figure 5.4A) in the purified active peak.

Electrospray ionization yielded an M+2H ion of m/z 925, confirming MALDI-TOF analysis (data not shown). This ion was fragmented using CID to yield doubly-charged fragments m/z 904.23, 864.27, and 762.71. These fragments corresponded with the loss of $-\text{CH}_2\text{CH}_2\text{NH}_2$, $-\text{PO}_3\text{CH}_2\text{CH}_2\text{NH}_2$, and $-\text{GalNac-PO}_3\text{CH}_2\text{CH}_2\text{NH}_2$ from the side chains, as will be discussed below in conjunction with NMR analyses.

5.3.2 Sequence analysis

Edman degradation of the active peak component yielded amino acid sequence of XEGPROGTKRP. Subsequent pretreatment of the peptide with acid and heat (19) de-blocked the N-terminus and allowed identification of a serinyl residue. Thus, the wilt-inducing active factor was found to comprise a peptide backbone (SEGPROGTKRP) having a predicted molecular mass of 1,197 Da. The lack of aromatic amino acid residues was consistent with the absence of absorbance by this material at 280 nm.

The recovered peptide sequence was used to query a database containing the *S. noctilio* venom gland transcriptome, and a single open reading frame (ORF) encoding this sequence was found in the most abundant transcript (database ID comp11322_c2_seq2). As assembled from Illumina sequencing data, this transcript was 397 base pairs (bp) in length and had a stop codon immediately following the terminal proline residue of the sequenced peptide (Figure 5.5A) and a 23 amino-acid signal peptide (Figure 5.5B). The encoding transcript was assembled from 24,015,498 Illumina reads of which 63,997 reads reached the 3' end and 46 reads reaching the 5' end (based on the inferred direction of translation). A 257-bp fragment of the inferred transcript that fully encompassed the predicted coding sequence was amplified from venom gland cDNAs using PCR. DNA sequencing of the 5'- and 3'-ends of the PCR amplicon corroborated the nucleotide sequence predicted from the Illumina assemblies.

5.3.3 NMR analysis

Purified noctilisin was subjected to a series of NMR analyses to further refine its structure. A 1D proton spectrum (Figure 5.6A) shows resonances characteristic of carbohydrates, such as the anomeric doublet at 4.862 ppm, in addition to those from peptide residues. Analyses of 2D COSY, TOCSY, ROESY, and HSQC data confirmed that the active factor was a glycopeptide containing the amino acid residues (SEGPROGTKRP) as well as two N-Acetyl- α -galactosamine residues (GalNAc) and two ethanolamine residues. Chemical shifts are listed in Table 5.1.

Chemical shifts for the protons associated with the hydroxyprolyl residue place the hydroxylation at the γ position (H1 4.57, C13 72.5 ppm). The NOESY spectrum (not shown) acquired from a sample in H₂O revealed seven amide protons with NOEs to α -protons of the previous residue, and so confirmed the sequence of fragments, S₁E₂G₃, P₄R₅, and hP₆G₇T₈K₉R₁₀. In addition, NOE crosspeaks were observed between the G₃ α and P₄ δ protons. Location of the α -GalNAc residues on the threonine and serine hydroxyls was confirmed by crosspeaks between the GalNAc H1 protons and the threonine and serine H β protons in the ROESY spectrum (Figure 5.6B). The ethanolamine residues did not yield detectable NOE signals with any other residues. However, the downfield position of the GalNAc H6 signals (H1 4.00, C13 67.8 ppm) coupled with the mass spectral data suggested a phosphate linkage to that position. This was confirmed by a 1D P³¹/H¹ correlation experiment (Figure 5.6C). Although the GalNAc-H4 proton signals were at the same position as the H6 signals, the large frequency spread (3.93-4.03 ppm) of the correlated protons compared to those of the GalNAc H4 protons, as well as the conventional chemical shifts of the H4 signals, indicated the ethanolamine substitutions were at the 6-position and not the 4-position. Scalar coupling constants were not generated. None of the NOE signals

were particularly suggestive of turns or helical regions, and since nearly all identifiable crosspeaks pointed to a most likely extended structure (Figure 5.7), significant secondary structural features for noctilisin did not appear to be supported.

5.3.4 Dose-dependent activity

To assign a rough specific activity value to noctilisin that would facilitate further work on its bioactivity, the molar absorptivity of the synthetic aglyconoctilisin was determined in water at 190 nm and compared to the theoretical absorptivity predicted for an 11-amino acid peptide at this signature wavelength for peptide bonds. The two were in close agreement, and since phosphoethanolamine and N-acetyl-galactosamine have negligible absorbance at 190 nm, concentrations of noctilisin in water were subsequently determined on the basis of a $\epsilon_{190} = 110,000$.

Using this molar absorptivity value, needle-response assays using *P. radiata* shoot tips indicated that 32 μM noctilisin contained activity roughly equivalent to a 20 mg/ml solution of whole (not heat-treated) *S. noctilio* venom in water (Figure 5.8). Dose-dependent responses for the PR4 and TLP biomarker genes were measured versus water at six noctilisin concentrations in two different *P. radiata* genotypes of intermediate venom sensitivity; the results from individual genotypes were nearly identical, and thus were averaged (Figure 5.9). Expression of both biomarkers saturated with application of 50 μl of 26-32 μM noctilisin in water. Synthetic aglyconoctilisin applied at concentrations of 30 μM and 300 μM (approximately 1x and 10x saturating values for noctilisin) failed to induce biomarker expression (Figure 5.10). Thus, one or both of the O-linked glycans are requisite for noctilisin bioactivity.

5.4 Discussion

Noctilisin, the heat-stable factor from *S. noctilio* venom that causes needle wilt and induces defense gene expression in attacked conifers, is an 11-amino acid glycopeptide encoded by the most abundant transcript expressed in venom gland tissues of this woodwasp. The ORF encoding noctilisin harbors two possible Met start codons. The longer sequence encodes a 34-amino acid peptide that is predicted by the SignalP 4.1 algorithm (23) to contain a secretion signal, as would be expected for a product that must accumulate extracellularly prior to sequestration in the venom reservoir (Figure 5.8). Besides cleavage to remove the inferred signal sequence, the noctilisin polypeptide backbone is post-translationally modified by hydroxylation of the Pro6 residue. NMR revealed that hydroxylation occurs at the C4 (γ) position, which favors a *trans* conformation and likely leads the peptide to assume an overall extended structure (24). Hydroxyprolyl residues are common in bioactive peptides and are often crucial for activity (25). Examples include Head Peptide I, a 10-amino acid neuropeptide in the mosquito, *Aedes aegypti*, where substitution of proline for hydroxyproline eliminated the peptide's effect on host-seeking behavior (26). In plants, hydroxyprolyl residues are critical to the function of several secreted peptide hormones, including CLV3, CLE2, and CEP1 (25). It remains to be seen whether hydroxylation of the prolyl residue in noctilisin is critical for the effects this peptide has in pine.

In contrast, the post-translational glycosyl modifications that decorate the noctilisin polypeptide were shown to be critical for bioactivity in pine. Polypeptides, like noctilisin, in which O-linked glycans are coupled in an α -configuration to the β -hydroxyl groups of serinyl or threonyl residues, are classified as mucin-type glycoproteins (27). Although not as well studied and understood structurally and functionally as N-linked glycans, the O-linked glycans in mucins are involved in a wide variety of important biological processes (28,29). Mucin glycosylation is

initiated by attachment of a single α -N-acetyl-galactosamine residue to Ser or Thr residues through the action UDP:GalNAc–polypeptide:GalNAc transferases (29,30). The product of this reaction, which is the same single-sugar glycosyl structure found in noctilisin, is also known as the Thomsen nouveau (Tn)-antigen for its tendency to elicit a strong immune response in some organisms (31). In mammals, mucin biosynthesis proceeds through further elaboration of the Tn-antigen structure by attachment of up to 20 additional sugar residues (32), but less extensive structures appear to be the norm for insect mucins (27). However, in specific mammalian cell types or disease-states, including cancer, this extension process may be lacking or blocked leaving naked Tn-antigen structures that can serve as easily detected immunological markers (33). The structural information collected for noctilisin suggests that commercially available reagents for histochemical detection of Tn-antigen may prove useful for future studies of noctilisin mobility and localization in trees, particularly since plants do not produce Tn-antigen structures that might interfere with immunodetection (34,35).

Like antibodies against the Tn-antigen, certain galactose-specific jacalin-related lectins (gJRLs) show high binding affinity for the Tn-antigen and as a consequence have been adapted for use as histology reagents to detect its presence (36,37). Lectins are widely distributed in plants (38,39) where they have long been recognized for their role in plant defense (40-42). A JRL with affinity for the Tn-antigen was isolated from ground ivy (*Glechoma hederacea*) and shown to have insecticidal properties (43). Tn-antigens have been detected previously in mixtures of salivary proteins from certain paper wasp species (44). Our identification of Tn-antigen in noctilisin, another insect secretory product, lends support to the idea that JRLs recognizing Tn-antigen structures could provide important front line surveillance as part of the defense systems plants use to detect insect attack. Although gene models for JRLs appear in

recently released conifer genome sequences (45,46), there is as yet no direct evidence for JRLs that could bind Tn-antigen in any of the pine species targeted by *S. noctilio*. It also remains to be seen whether the complete loss of bioactivity observed for aglyconoctilisin reflects failure of the naked peptide to evoke a lectin-mediated response.

Phosphoethanolaminyl modification of GalNAc residues has rarely been detected in glycopeptides, and it is unclear whether this structural modification plays an important functional role in noctilisin activity. Modification of glycoproteins with phosphoethanolaminyl moieties was first seen in N-linked glycans attached to apolipoprotein III from the hemolymph of the migratory locust, *Locusta migratoria* (47). The presence of O-glycans modified with phosphoethanolaminyl groups was reported more recently for salivary proteins from the German yellowjacket, *Vespula germanica* (44). However, similar structures were not detected in salivary proteins from the European hornet, *Vespa crabro*, (48), indicating that this modification is not universal across hymenoptera. It will be interesting to test whether phosphoethanolaminyl modification of noctilisin glycosyl groups affects the binding of Tn-specific antibodies and lectins since altered binding could help modified glycopeptides evade lectin-based surveillance systems in target plants.

Noctilisin may elicit the responses observed in pine through interaction with the extensive collection of receptor proteins that provide surveillance for the plant immune system (49). Peptide signaling systems involving such receptors have received increasing attention over the past 20 years for their role in controlling such diverse processes as plant growth and development, self/non-self recognition, and host-pathogen interactions (50-56). Although many peptide signaling systems operate over the short distances between plant cells (57,58), there are peptides that travel long distance through the plant vasculature before eliciting responses (59,60).

For example, the ethylene-inducing xylanase (EIX), originally isolated from *Trichoderma viride* (61), is translocated through the xylem in tobacco and pepper plants (62) before binding a specific leucine-rich repeat receptor-like protein (LRR-RLP) that undergoes endocytosis and causes a hypersensitive response (63).

Alternative mechanisms for noctilisin action are suggested from comparative venomics (18). Hymenopteran venoms generally comprise a diversity of bioactive components, but peptide constituents can comprise as much as 70% of the dry weight in these venoms (64). Many individual bioactive peptides from bee and wasp venoms have been studied with respect to structure-function relationships as well as their pharmacological properties. For instance, melittin, a 26-amino acid peptide that accounts for nearly 50% of the dry weight of honeybee (*Apis mellifera*) venom, assumes a bent alpha-helical structure and acts as a chaotropic and cytolytic agent in cell membranes (65). Apamin and mast cell-degranulating (MCD) peptide are closely related 18- and 22-amino acid peptides from honeybee that form ring structures and cause membrane depolarization through interaction with potassium channels (66). Mastoparans are 14-amino acid linear, polycationic peptides common in the venom from social wasps that interact with G-protein receptors and stimulate exocytosis (67). While none of the aforementioned bee and wasp venom peptides require glycosylation for activity, formaecin I, a 16-amino acid peptide with antibacterial activity isolated from the bulldog ant, *Myrmecia gulosa*, is decorated with a single O-linked GalNAc residue and this glycosylation has been shown to be important for bioactivity (68).

A search of GenBank with the noctilisin amino acid sequence identified homologous sequence motifs in proteins from a wide variety of sources, but drosocin, a member of the O-glycosylated PRP-rich cationic antibacterial peptide (CAP) family was of particular interest (69).

Drosocin, which is also related to formaecin I, was initially isolated from the hemolymph of *Drosophila melanogaster* on the basis of its activity against certain species of Gram-negative bacteria (70,71). Drosocin, thus, functions as a component of *Drosophila*'s innate immune system (72). The predominant form of drosocin is a 19-amino acid peptide modified at Thr11 by an O-linked disaccharide composed of GalNAc and Gal residues (T-antigen), but a doubly glycosylated form having the T-antigen attached to both Ser7 and Thr11 was described more recently (73,74). Differently glycosylated versions of both drosocin and formaecin I were shown to vary in their antibacterial activities when tested against a panel of different bacterial species (75). Unlike melittin and other chaotropic venom peptides, which act by disrupting target cell membranes, drosocin, formaecin, and related CAPs function by entering bacterial cells through peptide uptake systems and interacting with a specific intracellular protein target, DnaK, in a stereospecific fashion (76). DnaK is a member of the Hsp70 family of heat-shock proteins, and has been described as the central hub of the protein chaperone network that assists proper protein folding and prevents protein aggregate formation in cells (77,78). The antimicrobial activity of drosocin-like CAPs requires binding to DnaK, and the toxicity of these peptides thus appears to arise from disruption of normal protein folding machinery inside the cell. Naturally occurring CAPs have been studied extensively for their potential use as novel pharmacological agents, and none has so far shown evidence for binding eukaryotic Hsp70 proteins or cytotoxicity in eukaryotic cells, even when introduced directly into the cytoplasm at high concentration (79). However, synthetic peptides that bind and inhibit mammalian Hsp70 proteins have been identified and are being tested for their potential as anticancer agents (80). There are no reports in the literature of specific peptide inhibitors for plant Hsp70 proteins, but disruption of plant Hsp70 expression results in profound defects in growth and development as well as blockage of

the hypersensitive response (81,82). Whether noctilisin can enter pine cells and interact with Hsp70 proteins in a fashion similar to drosocin and formaecin I remains to be tested.

Noctilisin is the first bioactive peptide isolated from a hymenopteran insect whose function it is to elicit a set of specific physiological responses in plants. Availability of the purified peptide and synthetic derivatives will make it possible to study its mechanism of action in pine, which should help us understand the basis for observed variations in response to noctilisin between pine species and individual genotypes (17). We hope that improved understanding of the system will facilitate breeding of pines to enhance specific resistance against attack from *S. noctilio*.

Acknowledgments

The authors would like to thank Kelly Zylstra and Victor Mastro at USDA-APHIS for supplying insects. We also thank Nanda Menon at the UGA Bioexpression and Fermentation Facility for advice and use of the HPLC equipment; Valeria Coscia and Bruce Johnson at Noramco, and Chung-Jui Tsai in the Warnell School of Forestry and Natural Resources at UGA for use of HPLC columns; Jodie Franklin in the Synthesis & Sequencing Facility at Johns Hopkins University for her help with protein sequencing and peptide synthesis work; and Steve Pettis in the Warnell greenhouse for care of hedged seedlings. This work benefitted from NMR facilities made available through the Resource for Integrated Glycotechnology, a biomedical technology resource supported by a grant from the National Institutes of General Medical Sciences, P41 GM103390. This research was supported by a USDA-NIFA Graduate Fellowship (2012-67011-19694) awarded to JMB, and McIntire-Stennis project GEOZ-0154-MS.

Table 5.1: Proton chemical shifts for noctilisin glycopeptide in D₂O at 25 °C. Values referenced from HDO peak set to 4.760 ppm.

	α	β	γ	δ	ϵ	NH
Ser1	4.302	3.958,4.180				
Glu2	4.470	1.977,2.140	2.519			8.848
Gly3	4.180,3.957					8.382
Pro4	4.410	1.876,2.237	1.87	3.595		
Arg5	4.587	1.72,1.81	1.70	3.20		8.477
Hyp6	4.546	2.045,2.339	4.577	3.769,3.825		
Gly7	4.173,3.905					8.552
Thr8	4.504	4.243	1.274			8.371
Lys9	4.343	1.660,1.760	1.389	1.634	2.592	8.440
Arg10	4.474	1.74,1.80	1.70	3.20		8.422
Pro11	4.390	1.990,2.309	1.87	3.826,3.836		
	H1	H2	H3	H4	H5	H6
GalNAc (Ser)	4.858	4.065	3.843	3.98	4.14	4.00
GalNAc (Thr)	4.856	4.133	3.839	3.98	4.05	4.00
NAc	2.009,1.978					
ethanolamine	4.074	3.245				

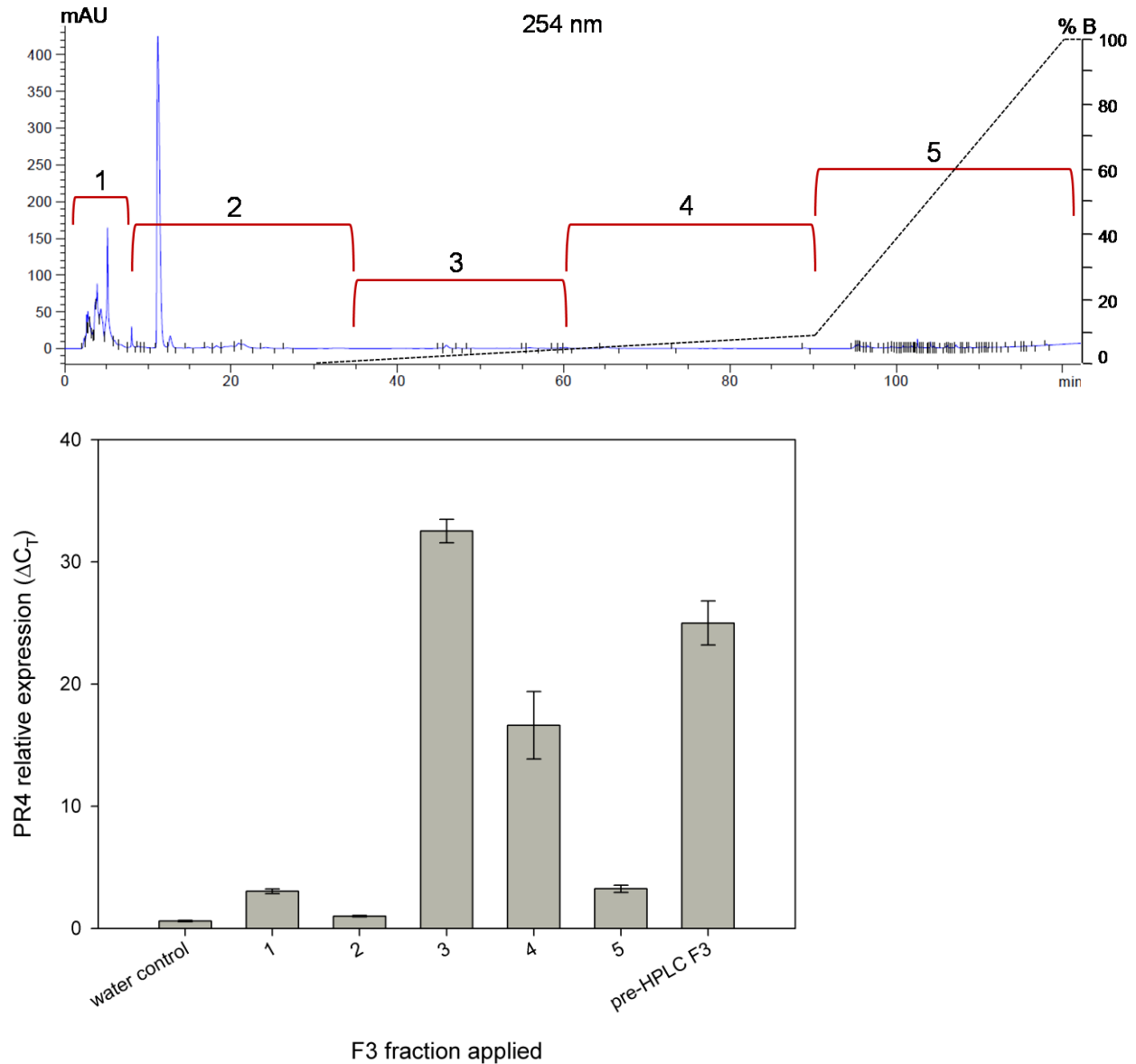


Figure 5.1: RP-HPLC fractionation of venom 10 kDa pass-through and bioassay evaluation. Compounds in a 500- μ L aliquot of *S. noctilio* venom fraction 3 (F3) were separated using C18 reversed-phase chromatography and following eluent absorbance at 254 nm. Eluent B was 0.04% TFA in acetonitrile. Noctilisin activity was essentially limited to the third and fourth fractions collected ($n = 3$).

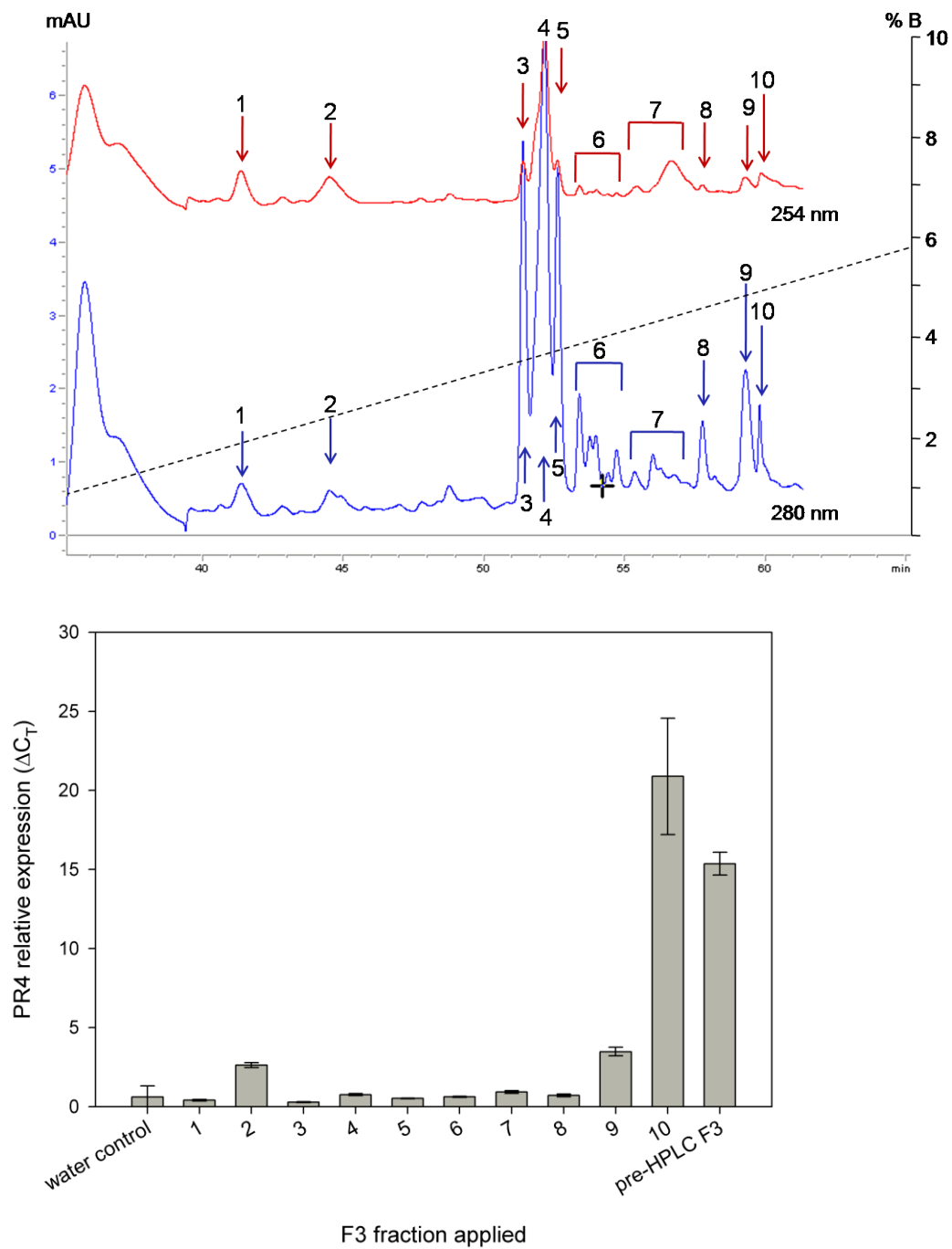


Figure 5.2: RP-HPLC fractionation of venom 10 kDa pass-through to single components and bioassay evaluation. Compounds in a 500- μ L aliquot of *S. noctilio* venom fraction 3 (F3) were separated using C18 reversed-phase chromatography and following eluent absorbance at both 254 and 280 nm. Eluent B was 0.04% TFA in acetonitrile. Activity was limited essentially to a single peak eluting at 59.6 min (n = 3).

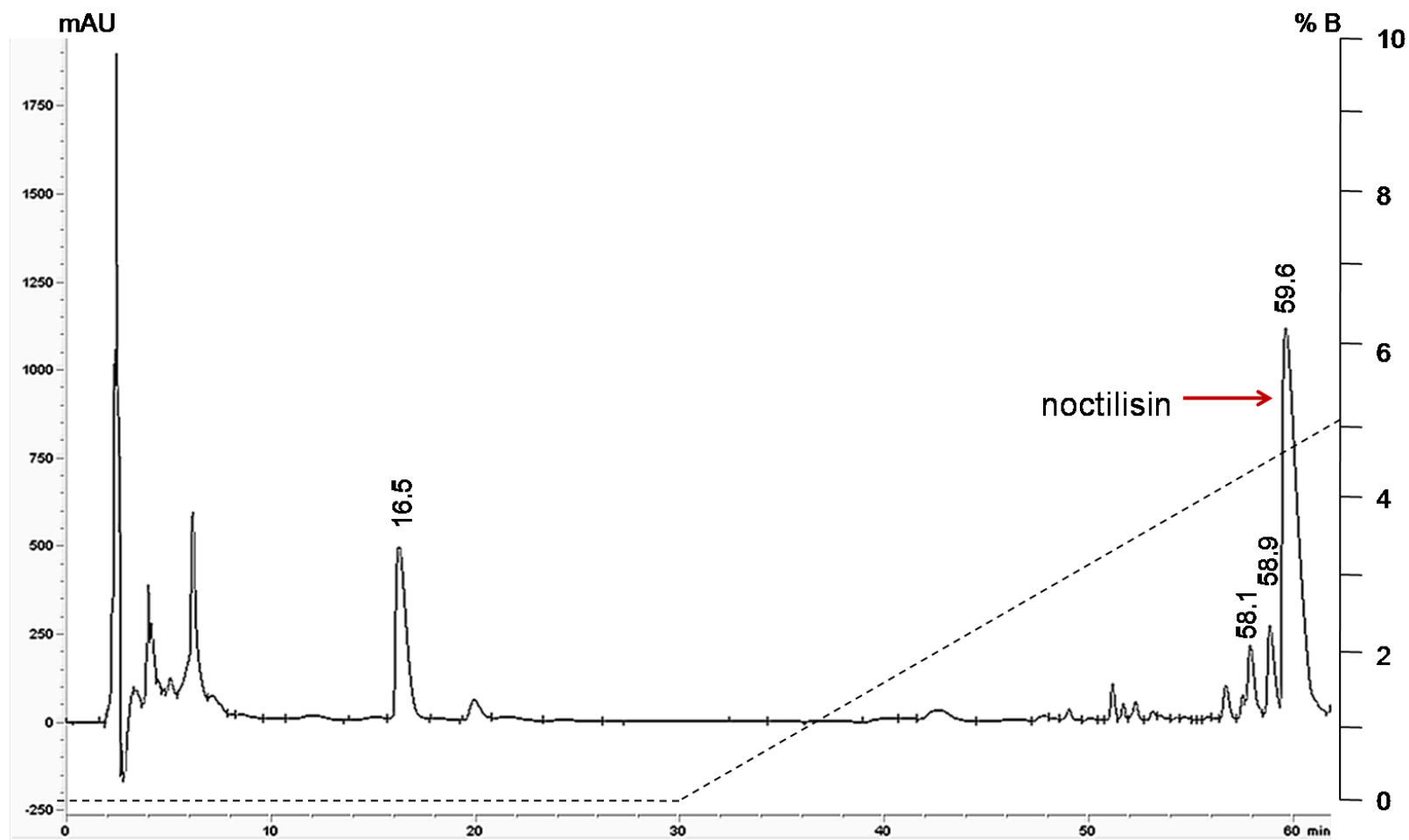


Figure 5.3: RP-HPLC purification of noctilisin. Compounds in a 500- μ L aliquot of *S. noctilio* venom fraction 3 (F3) were separated using C18 reversed-phase chromatography and following eluent absorbance at 210 nm. Noctilisin activity was limited to a single peak eluting at 59.6 min. Eluent B was 0.04% TFA in acetonitrile.

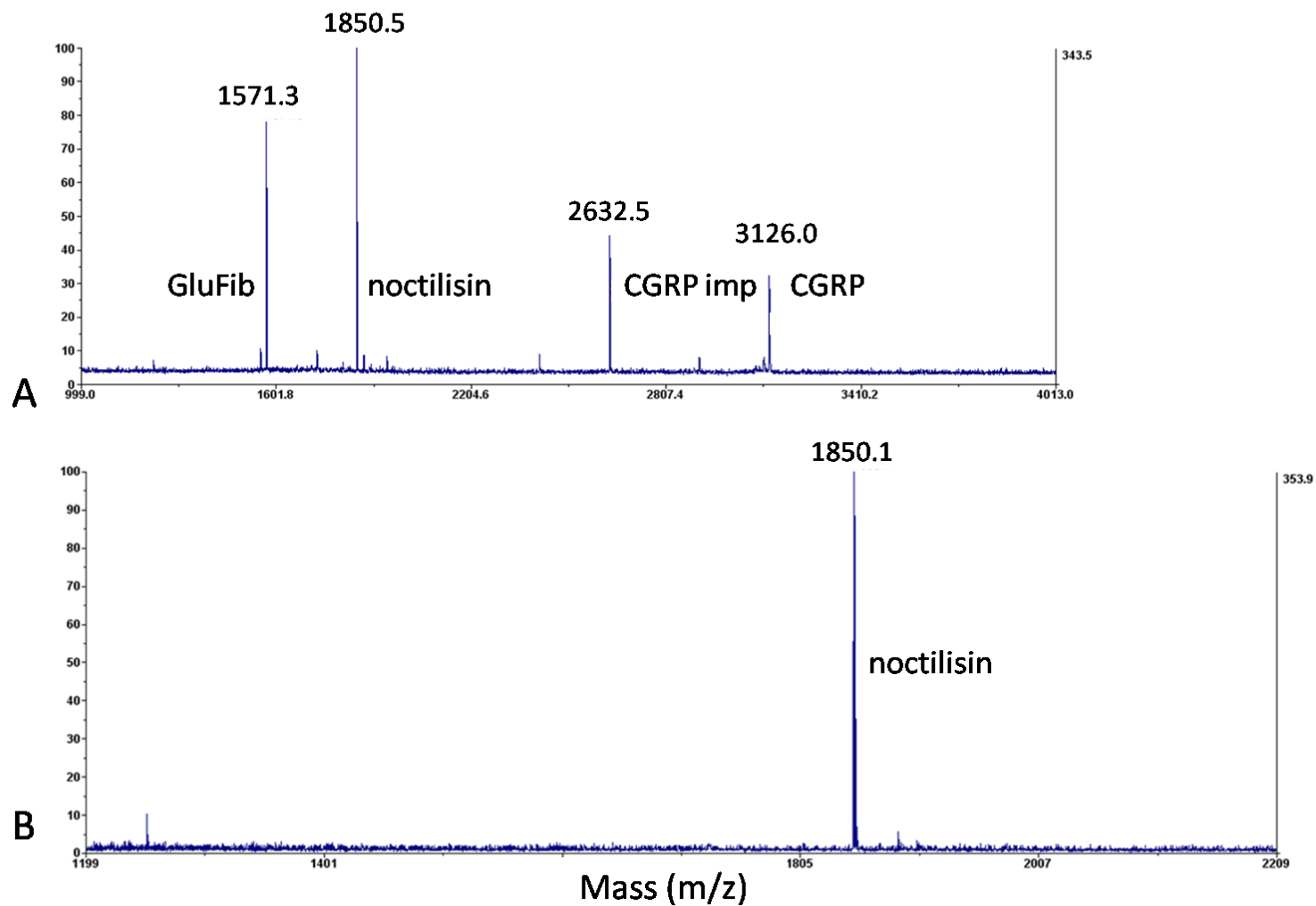
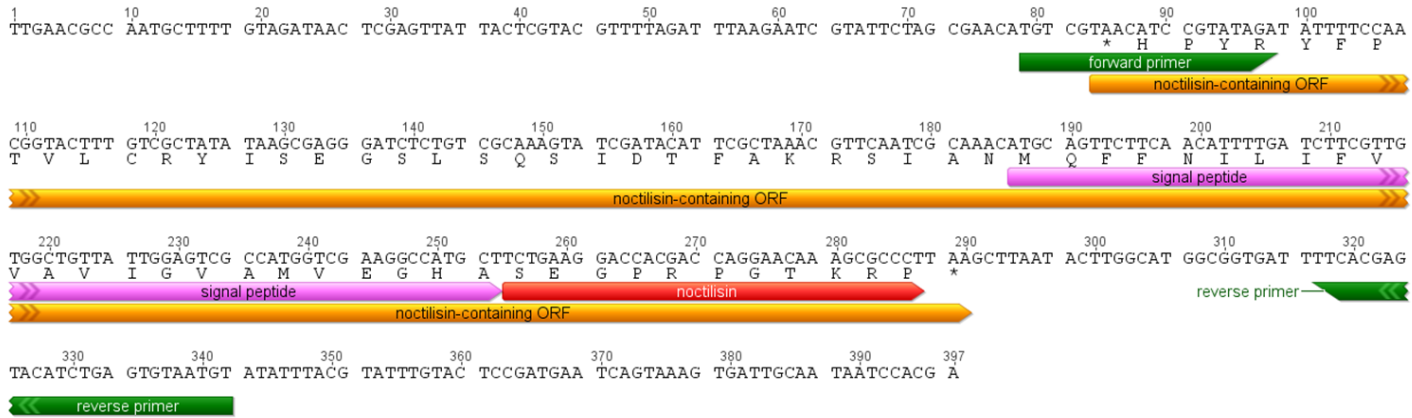
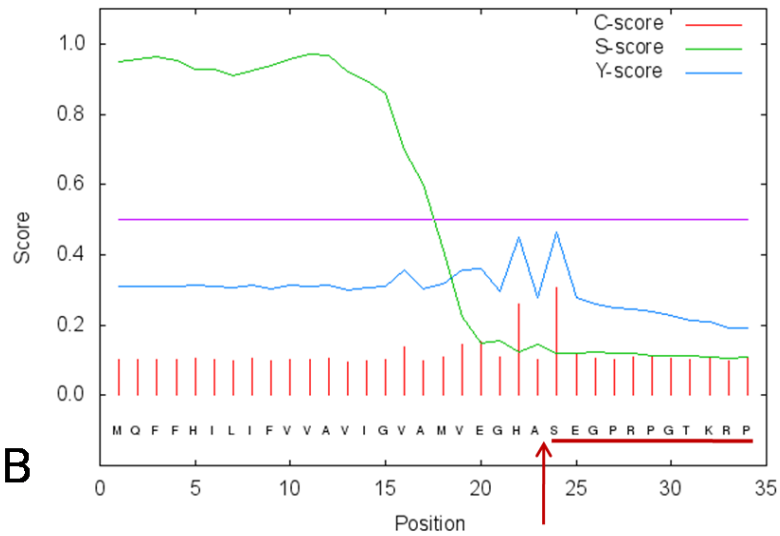


Figure 5.4: Molecular mass determination of noctilisin using MALDI-TOF/MS. Panel A depicts the purified glycopeptide along with two internal standards. The m/z 1571 peak is human [Glu]-1-fibrinopeptide B (GluFib, 1570.57 Da), and the m/z 3126 peak is calcitonin gene-related peptide fragment 8-37 (CGRP, 3125.59 Da). The additional peak at m/z 2633 in Panel A was introduced by the calcitonin gene-related peptide standard (CGRP imp). Panel B shows the glycopeptide alone.



A



B

Figure 5.5: Noctilisin transcript and amino acid sequence.

Figure 5.5: Noctilisin transcript and amino acid sequence. Panel A: the signal peptide sequence (pink bar) and the noctilisin peptide sequence (red bar) are contained within the open reading frame (orange bar). Sanger sequencing of venom gland cDNA amplified a 257-bp portion of the sequence (primers indicated by green bars). Image generated using Geneious, version 6.1 (Biomatters, Auckland, NZ). Panel B, signal peptide cleavage prediction between position 23 and 24 (red arrow) results in noctilisin peptide (red underline). Image generated using SignalP version 4.1 (23). C-score is the cleavage score, highest at the position after the cleavage site (here, at S). S-score distinguishes signal peptide from mature peptide sites in a protein. Y-score is the combined cleavage score, geometric mean of C and S score, accounting for the slope of the S-score. The purple line merely bisects the plot, and is included for evaluative perspective.

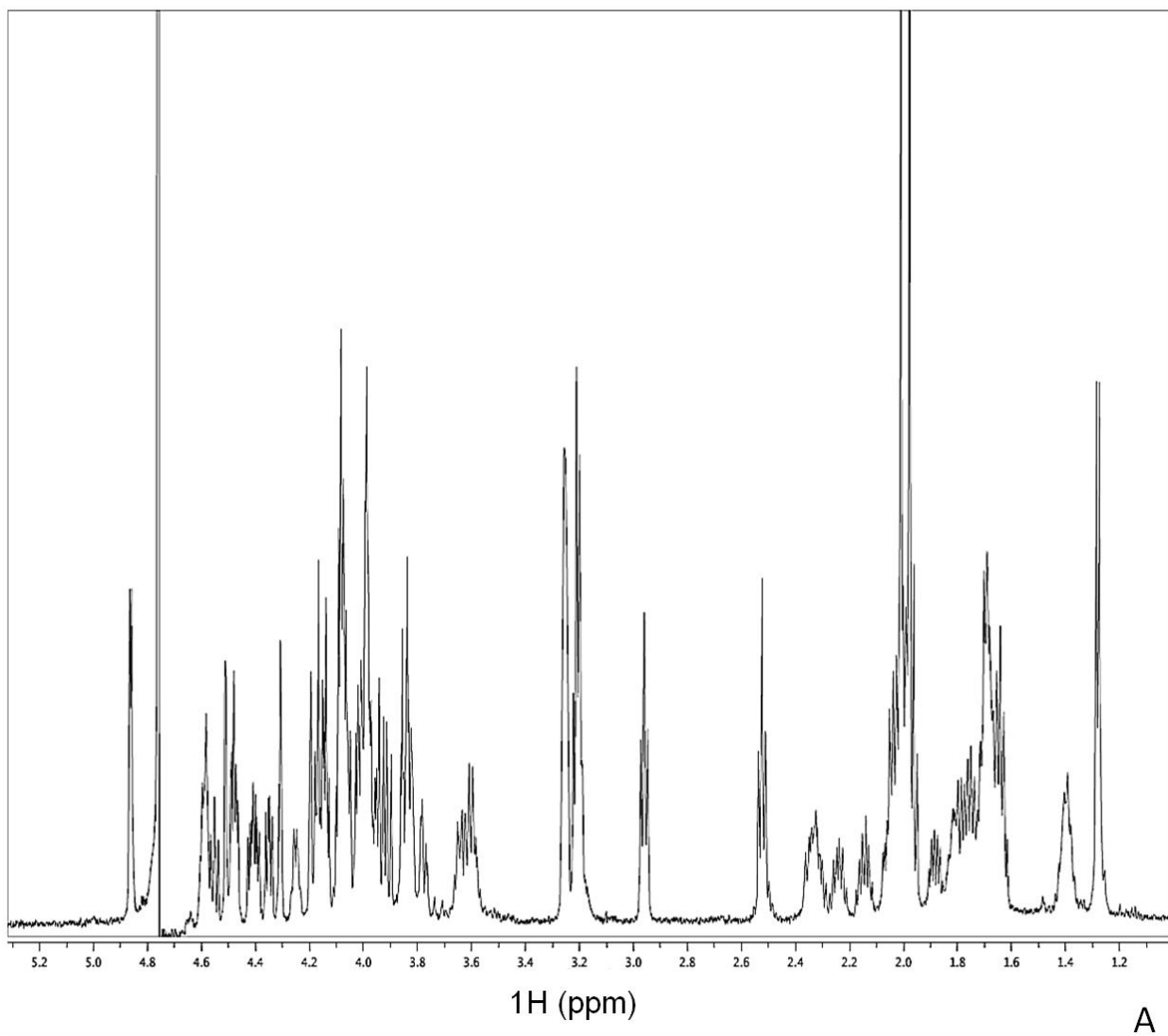
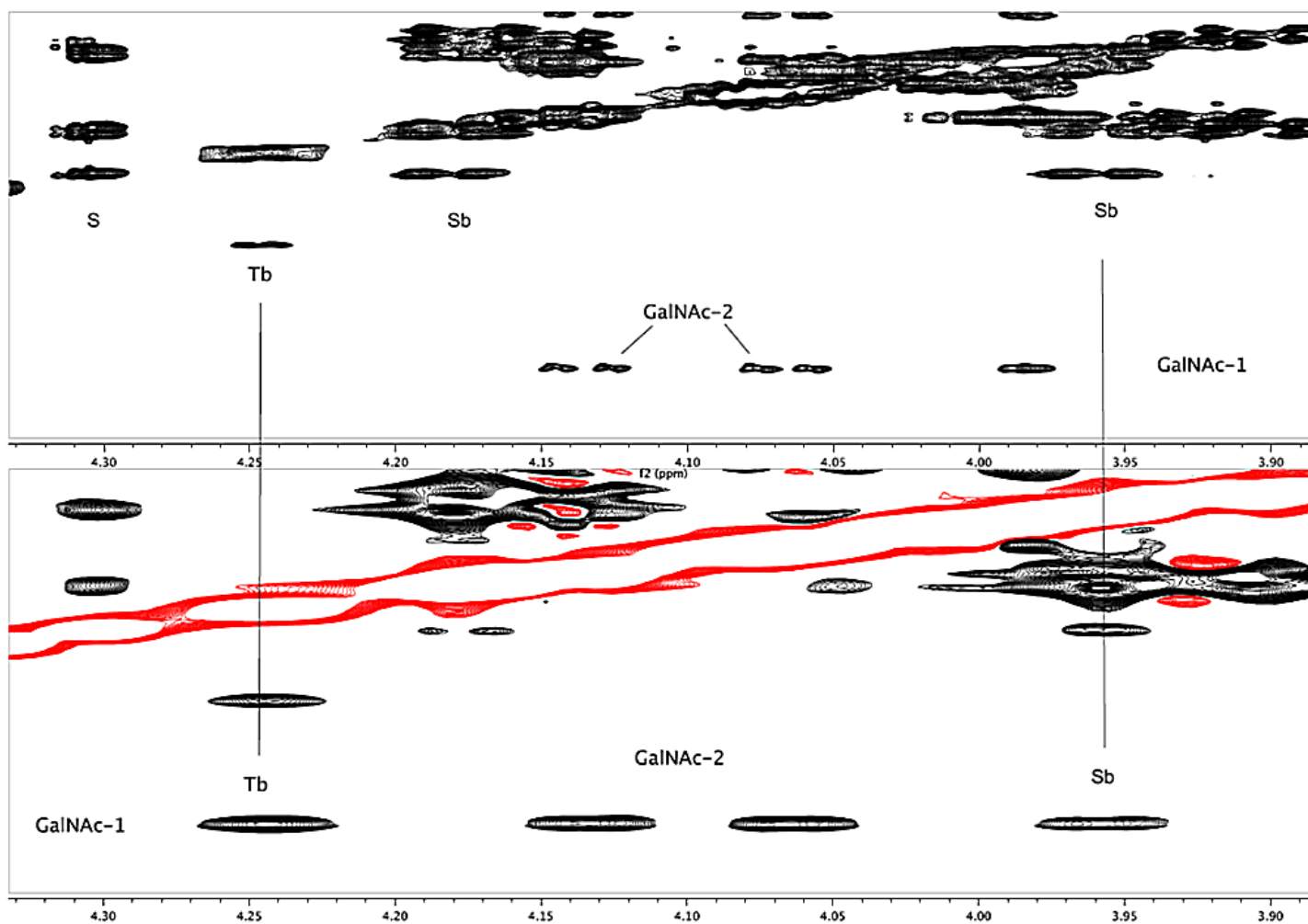


Figure 5.6A: NMR spectral analysis of purified noctilisin. Proton spectrum for noctilisin glycopeptide in D₂O at 25°C collected at 600 MHz.



B

Figure 5.6B: NMR spectral analysis of noctilisin glycans. Displayed are regions of TOCSY and ROESY spectra containing the crosspeaks between the GalNAc H1 protons and threonine and serine β -protons.

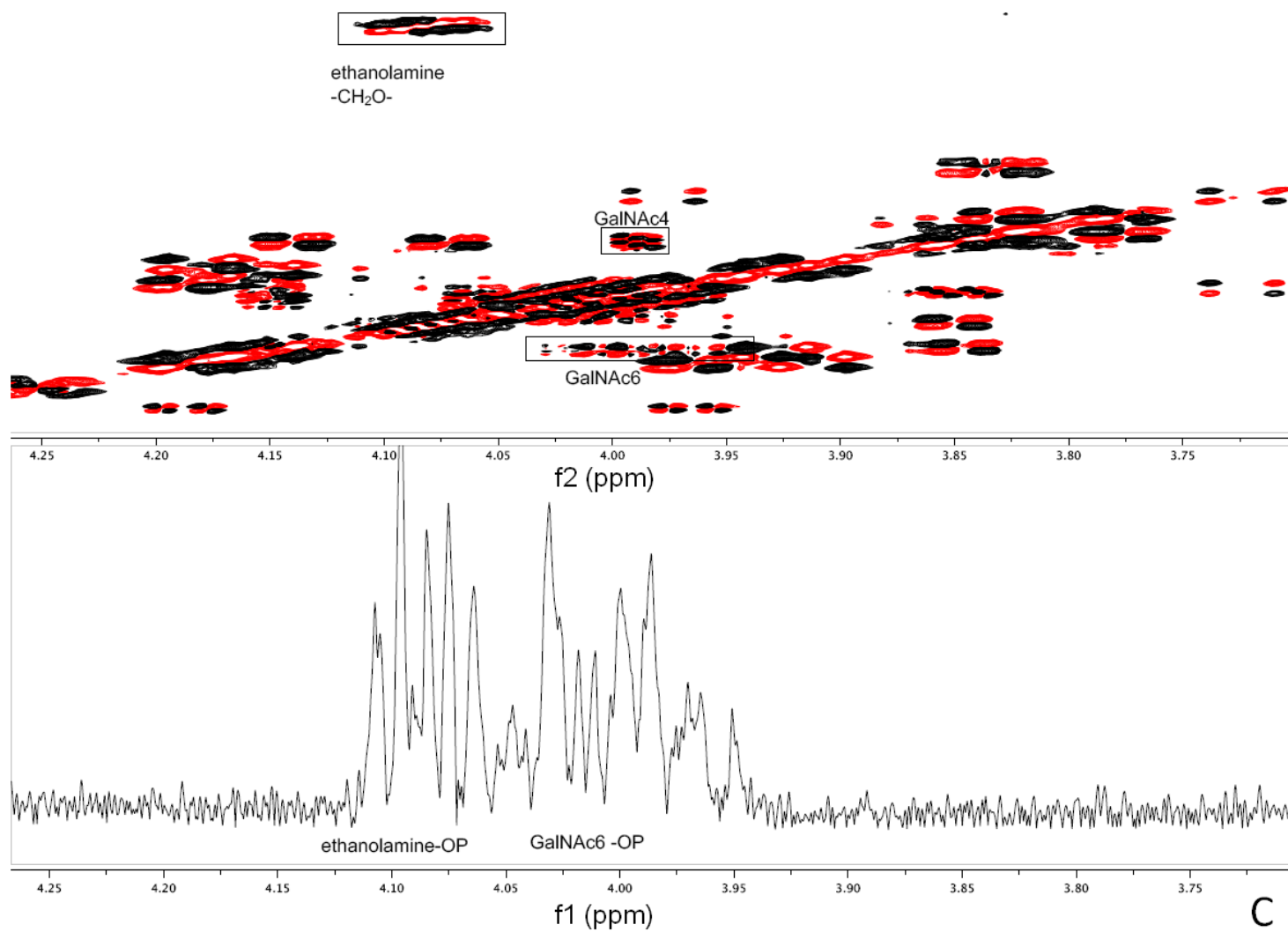


Figure 5.6C: 1D (¹H)³¹P HMQC spectrum showing signals from ethanolamine protons as well as GalNAc H6 protons coupled to phosphorous.

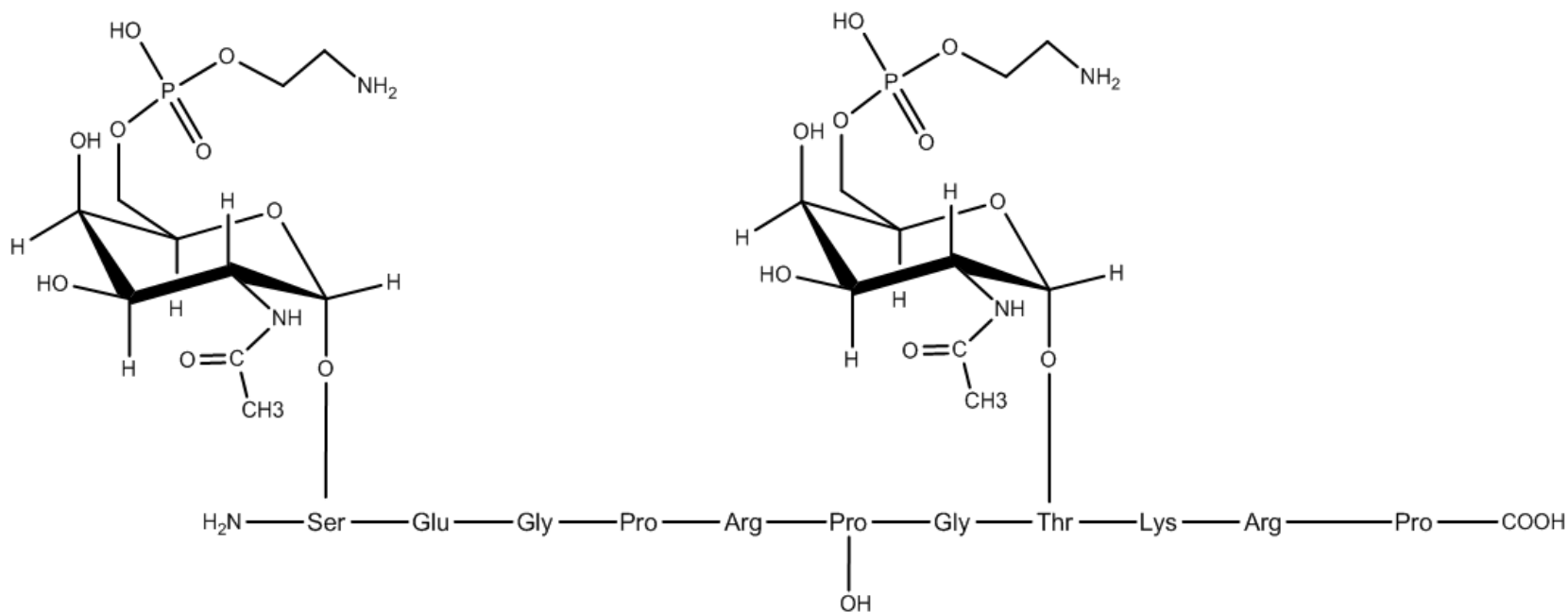


Figure 5.7: Noctilisin structure. Residues 1 and 8 of the peptide backbone are O-glycosylated through an α -linkage to N-acetylgalactosamine modified by attachment of a phosphoethanolamine moiety to the C6 oxygen. NOE data did not indicate turns or helical regions in the peptide backbone, and identifiable crosspeaks supported an extended structure overall. Figure created using ChemBioDraw software (PerkinElmer, Waltham, MA).

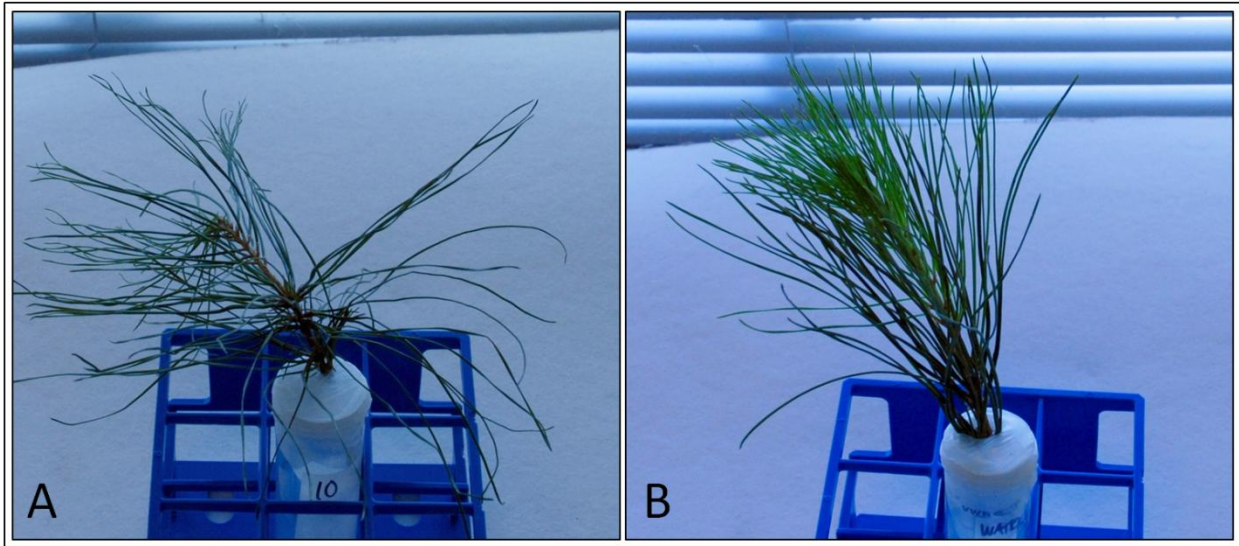


Figure 5.8: Typical pine shoot tip bioassay responses. Shoot tips from sensitive *P. radiata* plants were allowed to take up 50 μL of noctilisin in water through their cut ends. After uptake, shoot tips were transferred to water and incubated for ten days. Cut ends were trimmed every three days to minimize xylem blockage. Panel A, 97 μM purified noctilisin; panel B, water control. Responses have been reproduced in two other *P. radiata* seedling rated to be of high or intermediate sensitivity to *S. noctilio* venom.

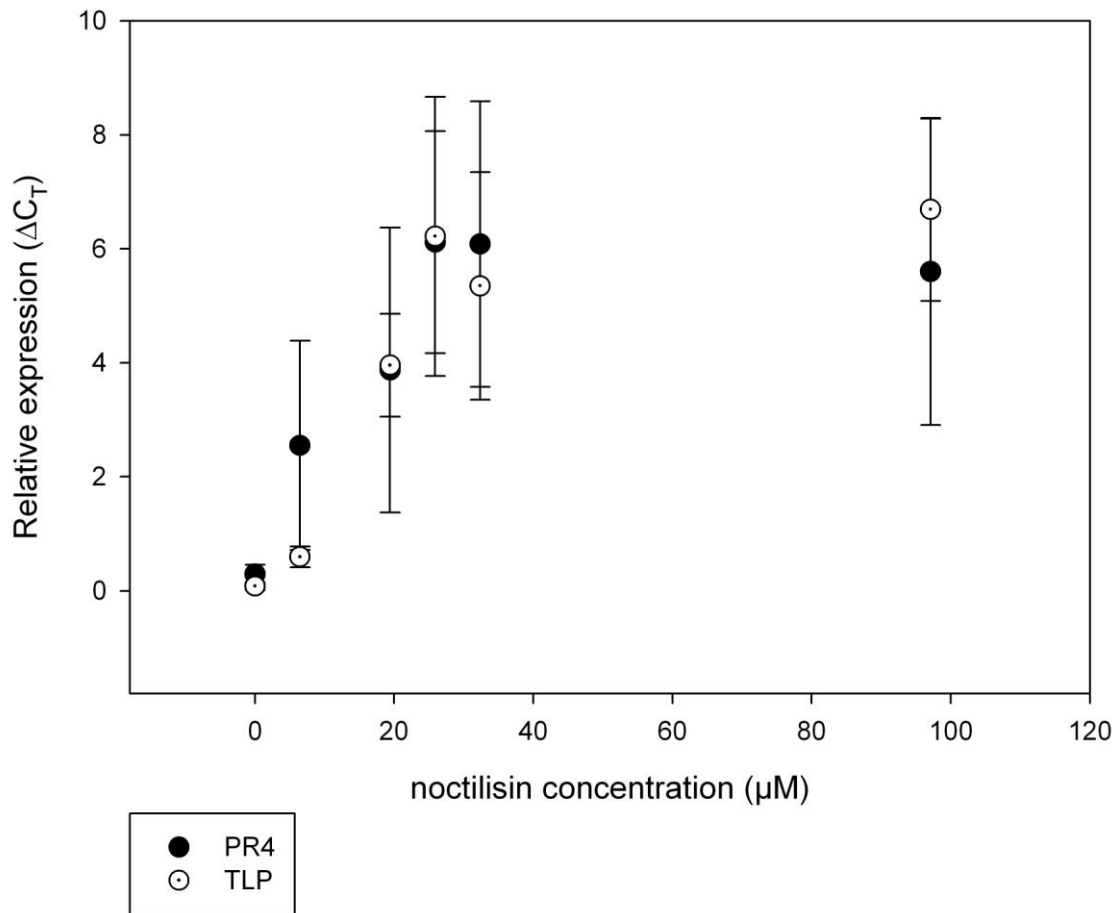


Figure 5.9: Dose-dependence of noctilisin bioactivity via qRT-PCR. Shoot tips from two *P. radiata* genotypes with known sensitivity to *S. noctilio* venom were administered 50 μL of noctilisin at the given concentrations in water. Expression levels of the PR4 and TLP biomarker genes were assessed in comparison to the ACT control gene after 24 h. For each data point, n = 6 (2 genotypes x 3 shoot tips).

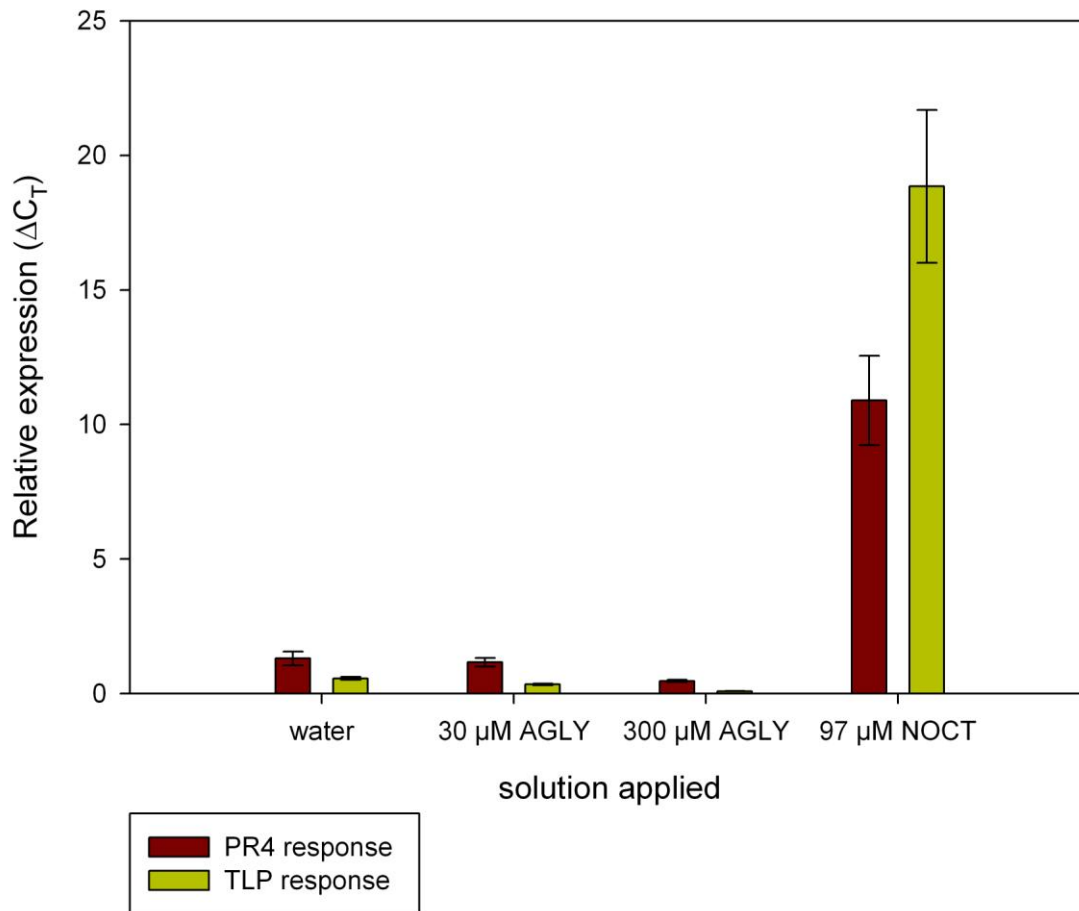


Figure 5.10: Dependence via qRT-PCR of noctilisin bioactivity on glycosyl modification. Shoot tips from a single *P. radiata* tree were administered 50 μ L of noctilisin (NOCT) or aglyconoctilisin (AGLY) at the given concentrations in water, then transferred to water. Expression levels of the PR4 and TLP biomarker genes were assessed in comparison to the ACT control gene after 24 h. For each data point, n = 3.

References

1. Slippers, B., de Groot, P., and Wingfield, M. J. (eds). (2012) *The Sirex Woodwasp and its Fungal Symbiont*, Springer, New York
2. Dodds, K. J., de Groot, P., and Orwig, D. A. (2010) The impact of *Sirex noctilio* in *Pinus resinosa* and *Pinus sylvestris* stands in New York and Ontario. *Canadian Journal of Forest Research* **40**, 212-223
3. Dodds, K. J., and Groot, P. (2012) *Sirex*, Surveys and Management: Challenges of having *Sirex noctilio* in North America. in *The Sirex Woodwasp and its Fungal Symbiont*: (Slippers, B., de Groot, P., and Wingfield, M. J. eds.), Springer Netherlands. pp 265-286
4. Rawlings, G. B. (1948) Recent observations on the *Sirex noctilio* population in *Pinus radiata* forests in New Zealand. *New Zealand Journal of Forestry* **5**, 411-421
5. Fong, L. K., and Crowden, R. K. (1973) Physiological effects of mucus from wood wasp, *Sirex noctilio* F., on foliage of *Pinus radiata* D. Don. *Aust. J. Biol. Sci.* **26**, 365-378
6. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .2. Effects of *S. noctilio* mucus. *Aust. J. Biol. Sci.* **22**, 1153-1161
7. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .I. Effects of symbiotic fungus *Amylostereum* sp. (Thelophoraceae). *Aust. J. Biol. Sci.* **22**, 915-924
8. Bordeaux, J. M., and Dean, J. F. D. (2012) Susceptibility and response of pines to *Sirex noctilio*. in *Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest* (Slippers, B., DeGroot, P., and Wingfield, M. J. eds.), Springer, New York. pp 31-50

9. Wong, L. K., and Crowden, R. K. (1976) Preliminary studies on mucus secretion of wood wasp, *Sirex noctilio* F .1. Physicochemical and biochemical properties. *Aust. J. Biol. Sci.* **29**, 21-32
10. Moreau, S. J. M. (2013) “It stings a bit but it cleans well”: venoms of Hymenoptera and their antimicrobial potential. *J. Insect Physiol.* **59**, 186-204
11. Colinet, D., Mathe-Hubert, H., Allemand, R., Gatti, J. L., and Poirie, M. (2013) Variability of venom components in immune suppressive parasitoid wasps: From a phylogenetic to a population approach. *J. Insect Physiol.* **59**, 205-212
12. Asgari, S., and Rivers, D. B. (2011) Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. in *Annual Review of Entomology, Vol 56* (Berenbaum, M. R., Carde, R. T., and Robinson, G. E. eds.), Annual Reviews, Palo Alto. pp 313-335
13. Danneels, E. L., Rivers, D. B., and de Graaf, D. C. (2010) Venom proteins of the parasitoid wasp *Nasonia vitripennis*: recent discovery of an untapped pharmacopee. *Toxins* **2**, 494-516
14. Piek, T. (ed) (1986) *Venoms of the Hymenoptera: biochemical, pharmacological and behavioural aspects.*, Academic Press, London
15. Boros, C. B. (1968) *The relationship between the woodwasp Sirex noctilio F. and the wood-rot fungus Amylostereum sp.* Master of Agricultural Science M. S., University of Adelaide
16. Coutts, M. P. (1970) The physiological effects of the mucus secretion of *Sirex noctilio* on *Pinus radiata*. *Aust. For. Res.* **4**, 23-26

17. Bordeaux, J. M., Lorenz, W. W., and Dean, J. F. D. (2012) Biomarker genes highlight intraspecific and interspecific variations in the responses of *Pinus taeda* L. and *Pinus radiata* D. Don to *Sirex noctilio* F. acid gland secretions. *Tree Physiol.* **32**, 1302-1312
18. Vetter, I., Davis, J. L., Rash, L. D., Anangi, R., Mobli, M., Alewood, P. F., Lewis, R. J., and King, G. F. (2011) Venomics: a new paradigm for natural products-based drug discovery. *Amino Acids* **40**, 15-28
19. Wellner, D., Panneerselvam, C., and Horecker, B. L. (1990) Sequencing of peptides and proteins with blocked N-terminal amino acids N-acetylserine or N-acetylthreonine. *Proc. Natl. Acad. Sci. USA* **87**, 1947-1949
20. Rozen, S., and Skaletsky, H. J. (2000) Primer3 on the WWW for general users and for biologist programmers. in *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (S., K., and S., M. eds.), Humana Press, Totowa, NJ. pp 365-386
21. Kingston, R. L., Baase, W. A., and Gay, L. S. (2005) Characterization of nucleocapsid binding by the measles virus and mumps virus phosphoproteins. *Journal of Virology* **79**, 10097-10097
22. Hennessey, J. P., and Johnson, W. C. (1981) Information content in the circular dichroism of proteins. *Biochemistry* **20**, 1085-1094
23. Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**, 785-786
24. Gorres, K. L., and Raines, R. T. (2010) Prolyl 4-hydroxylase. *Crit. Rev. Biochem. Mol. Biol.* **45**, 106-124
25. Matsubayashi, Y. (2011) Post-translational modifications in secreted peptide hormones in plants. *Plant Cell Physiol.* **52**, 5-13

26. Brown, M. R., Klowden, M. J., Crim, J. W., Young, L., Shrouder, L. A., and Lea, A. O. (1994) Endogenous regulation of mosquito host-seeking behavior by a neuropeptide. *J. Insect Physiol.* **40**, 399-406
27. Tian, E., and Ten Hagen, K. G. (2009) Recent insights into the biological roles of mucin-type O-glycosylation. *Glycoconjugate Journal* **26**, 325-334
28. Hanisch, F.-G. (2001) O-glycosylation of the mucin type. *Biol. Chem.* **382**, 143-149
29. Hang, H. C., and Bertozzi, C. R. (2005) The chemistry and biology of mucin-type O-linked glycosylation. *Bioorganic Med. Chem.* **13**, 5021-5034
30. Bennett, E. P., Mandel, U., Clausen, H., Gerken, T. A., Fritz, T. A., and Tabak, L. A. (2012) Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family. *Glycobiol.* **22**, 736-756
31. Ju, T., Otto, V. I., and Cummings, R. D. (2011) The Tn antigen—structural simplicity and biological complexity. *Angewandte Chemie International Edition* **50**, 1770-1791
32. Grogan, M. J., Pratt, M. R., Marcaurelle, L. A., and Bertozzi, C. R. (2002) Homogeneous glycopeptides and glycoproteins for biological investigation. *Ann. Rev. Biochem.* **71**, 593-634
33. Ju, T., and Cummings, R. D. (2005) Protein glycosylation: chaperone mutation in Tn syndrome. *Nature* **437**, 1252-1252
34. Vandenborre, G., Smagghe, G., Ghesquière, B., Menschaert, G., Nagender Rao, R., Gevaert, K., and Van Damme, E. J. M. (2011) Diversity in protein glycosylation among insect species. *PLoS One* **6**, e16682

35. Yang, Z., Drew, D. P., Jørgensen, B., Mandel, U., Bach, S. S., Ulvskov, P., Levery, S. B., Bennett, E. P., Clausen, H., and Petersen, B. L. (2012) Engineering mammalian mucin-type O-glycosylation in plants. *J. Biol. Chem.* **287**, 11911-11923
36. Tachibana, K., Nakamura, S., Wang, H., Iwasaki, H., Tachibana, K., Maebara, K., Cheng, L., Hirabayashi, J., and Narimatsu, H. (2006) Elucidation of binding specificity of jacalin toward O-glycosylated peptides: quantitative analysis by frontal affinity chromatography. *Glycobiol.* **16**, 46-53
37. Rougé, P., Peumans, W., Damme, E. M., Barre, A., Singh, T., Wu, J., and Wu, A. (2011) Glycotope Structures and Intramolecular Affinity Factors of Plant Lectins for Tn/T Antigens. in *The Molecular Immunology of Complex Carbohydrates-3* (Wu, A. M. ed.), Springer US. pp 143-154
38. Raval, S., Gowda, S. B., Singh, D. D., and Chandra, N. R. (2004) A database analysis of jacalin-like lectins: sequence-structure-function relationships. *Glycobiol.* **14**, 1247-1263
39. Sharon, N., and Lis, H. (2004) History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiol.* **14**, 53R-62R
40. Vandenborre, G., Smagghe, G., and Van Damme, E. J. M. (2011) Plant lectins as defense proteins against phytophagous insects. *Phytochemistry* **72**, 1538-1550
41. Xiang, Y., Song, M., Wei, Z., Tong, J., Zhang, L., Xiao, L., Ma, Z., and Wang, Y. (2011) A jacalin-related lectin-like gene in wheat is a component of the plant defence system. *J. Exp. Bot.* **62**, 5471-5483
42. Michiels, K., Van Damme, E. J. M., and Smagghe, G. (2010) Plant-insect interactions: what can we learn from plant lectins? *Arch. Insect Biochem. Physiol.* **73**, 193-212

43. Wang, W. F., Hause, B., Peumans, W. J., Smagghe, G., Mackie, A., Fraser, R., and Van Damme, E. J. M. (2003) The Tn antigen-specific lectin from ground ivy is an insecticidal protein with an unusual physiology. *Plant Physiol.* **132**, 1322-1334
44. Maes, E., Garénaux, E., Strecker, G., Leroy, Y., Wieruszeski, J.-M., Brassart, C., and Guérardel, Y. (2005) Major O-glycans from the nest of *Vespula germanica* contain phospho-ethanolamine. *Carbohydr. Res.* **340**, 1852-1858
45. Nystedt, B., Street, N. R., Wetterbom, A., Zuccolo, A., Lin, Y. C., Scofield, D. G., Vezzi, F., Delhomme, N., Giacomello, S., Alexeyenko, A., Vicedomini, R., Sahlin, K., Sherwood, E., Elfstrand, M., Gramzow, L., Holmberg, K., Hallman, J., Keech, O., Klasson, L., Koriabine, M., Kucukoglu, M., Kaller, M., Luthman, J., Lysholm, F., Niittyla, T., Olson, A., Rilakovic, N., Ritland, C., Rossello, J. A., Sena, J., Svensson, T., Talavera-Lopez, C., Theissen, G., Tuominen, H., Vanneste, K., Wu, Z. Q., Zhang, B., Zerbe, P., Arvestad, L., Bhalerao, R., Bohlmann, J., Bousquet, J., Gil, R. G., Hvidsten, T. R., de Jong, P., MacKay, J., Morgante, M., Ritland, K., Sundberg, B., Thompson, S. L., Van de Peer, Y., Andersson, B., Nilsson, O., Ingvarsson, P. K., Lundeberg, J., and Jansson, S. (2013) The Norway spruce genome sequence and conifer genome evolution. *Nature* **497**, 579-584
46. Birol, I., Raymond, A., Jackman, S. D., Pleasance, S., Coope, R., Taylor, G. A., Saint Yuen, M. M., Keeling, C. I., Brand, D., Vandervalk, B. P., Kirk, H., Pandoh, P., Moore, R. A., Zhao, Y. J., Mungall, A. J., Jaquish, B., Yanchuk, A., Ritland, C., Boyle, B., Bousquet, J., Ritland, K., MacKay, J., Bohlmann, J., and Jones, S. J. M. (2013) Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data. *Bioinformatics* **29**, 1492-1497

47. Hard, K., Van Doorn, J. M., Thomas-Oates, J. E., Kamerling, J. P., and Van der Horst, D. J. (1993) Structure of the Asn-linked oligosaccharides of apolipoprotein III from the insect *Locusta migratoria*. Carbohydrate-linked 2-aminoethylphosphonate as a constituent of a glycoprotein. *Biochemistry* **32**, 766-775
48. Garenaux, E., Maes, E., Levêque, S., Brassart, C., and Guerardel, Y. (2011) Structural characterization of complex O-linked glycans from insect-derived material. *Carbohydr. Res.* **346**, 1093-1104
49. Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006) Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell* **124**, 803-814
50. Germain, H., Chevalier, E., and Matton, D. P. (2006) Plant bioactive peptides: an expanding class of signaling molecules. *Canadian Journal of Botany-Revue Canadienne De Botanique* **84**, 1-19
51. Jones, J. D. G., and Dangl, J. L. (2006) The plant immune system. *Nature* **444**, 323-329
52. Matsubayashi, Y., and Sakagami, Y. (2006) Peptide hormones in plants. in *Annu. Rev. Plant. Biol.* pp 649-674
53. Katsir, L., Davies, K. A., Bergmann, D. C., and Laux, T. (2011) Peptide signaling in plant development. *Current Biology* **21**, R356-R364
54. Yamaguchi, Y., and Huffaker, A. (2011) Endogenous peptide elicitors in higher plants. *Current Opinion in Plant Biology* **14**, 351-357
55. Aalen, R. B. (2013) Maturing peptides open for communication. *J. Exp. Bot.* **64**, 5231-5235
56. Albert, M. (2013) Peptides as triggers of plant defence. *J. Exp. Bot.* **64**, 5269-5279

57. Marshall, E., Costa, L. M., and Gutierrez-Marcos, J. (2011) Cysteine-rich peptides (CRPs) mediate diverse aspects of cell-cell communication in plant reproduction and development. *J. Exp. Bot.* **62**, 1677-1686
58. Murphy, E., Smith, S., and De Smet, I. (2012) Small signaling peptides in *Arabidopsis* development: how cells communicate over a short distance. *Plant Cell* **24**, 3198-3217
59. Neumann, P. M. (2007) Evidence for long-distance xylem transport of signal peptide activity from tomato roots. *J. Exp. Bot.* **58**, 2217-2223
60. Okamoto, S., Shinohara, H., Mori, T., Matsubayashi, Y., and Kawaguchi, M. (2013) Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase. *Nature Communications* **4**
61. Dean, J. F. D., and Anderson, J. D. (1991) Ethylene biosynthesis-inducing xylanase 2: purification and physical characterization of the enzyme produced by *Trichoderma viride*. *Plant Physiol.* **95**, 316-323
62. Bailey, B. A., Taylor, R., Dean, J. F. D., and Anderson, J. D. (1991) Ethylene biosynthesis-inducing endoxylanase is translocated through the xylem of *Nicotiana tabacum* cv *xanthi* plants. *Plant Physiol.* **97**, 1181-1186
63. Ron, M., and Avni, A. (2004) The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant Cell* **16**, 1604-1615
64. Palma, M. S. (2006) Insect venom peptides. in *Handbook of Biologically Active Peptides* (Kastin, A. J. ed.), Academic Press, New York. pp 389-396
65. Raghuraman, H., and Chattopadhyay, A. (2007) Melittin: a membrane-active peptide with diverse functions. *Bioscience Reports* **27**, 189-223

66. Froy, O., and Gurevitz, M. (1998) Membrane potential modulators: a thread of scarlet from plants to humans. *Faseb Journal* **12**, 1793-1796
67. Higashijima, T., Uzu, S., Nakajima, T., and Ross, E. M. (1988) Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G-proteins). *J. Biol. Chem.* **263**, 6491-6494
68. Mackintosh, J. A., Veal, D. A., Beattie, A. J., and Gooley, A. A. (1998) Isolation from an ant *Myrmecia gulosa* of two inducible O-glycosylated proline-rich antibacterial peptides. *J. Biol. Chem.* **273**, 6139-6143
69. Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* **3**, 238-250
70. Bulet, P., Dimarcq, J. L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Vandorsselaer, A., and Hoffmann, J. A. (1993) A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution. *J. Biol. Chem.* **268**, 14893-14897
71. Bennett, C. S., Payne, R. J., Koeller, K. M., and Wong, C.-H. (2008) Biologically Relevant Glycopeptides: Synthesis and Applications. in *Glycoscience* (Fraser-Reid, B., Tatsuta, K., and Thiem, J. eds.), Springer Berlin Heidelberg. pp 1795-1857
72. Hoffmann, J. A., and Reichhart, J. M. (2002) *Drosophila* innate immunity: an evolutionary perspective. *Nature Immunology* **3**, 121-126
73. Levy, F., Rabel, D., Charlet, M., Bulet, P., Hoffmann, J. A., and Ehret-Sabatier, L. (2004) Peptidomic and proteomic analyses of the systemic immune response of *Drosophila*. *Biochimie* **86**, 607-616

74. Rabel, D., Charlet, M., Ehret-Sabatier, L., Cavicchioli, L., Cudic, M., Otvos, L., and Bulet, P. (2004) Primary structure and in vitro antibacterial properties of the *Drosophila melanogaster* attacin C pro-domain. *J. Biol. Chem.* **279**, 14853-14859
75. Talat, S., Thiruvikraman, M., Kumari, S., and Kaur, K. J. (2011) Glycosylated analogs of formaecin I and drosocin exhibit differential pattern of antibacterial activity. *Glycoconjugate Journal* **28**, 537-555
76. Otvos, L., O, I., Rogers, M. E., Consolvo, P. J., Condie, B. A., Lovas, S., Bulet, P., and Blaszczyk-Thurin, M. (2000) Interaction between heat shock proteins and antimicrobial peptides. *Biochemistry* **39**, 14150-14159
77. Mayer, M. P., and Bukau, B. (2005) Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences* **62**, 670-684
78. Calloni, G., Chen, T., Schermann, S. M., Chang, H. C., Genevaux, P., Agostini, F., Tartaglia, G. G., Hayer-Hartl, M., and Hartl, F. U. (2012) DnaK functions as a central hub in the *E. coli* chaperone network. *Cell Reports* **1**, 251-264
79. Hansen, A., Schafer, I., Knappe, D., Seibel, P., and Hoffmann, R. (2012) Intracellular toxicity of proline-rich antimicrobial peptides shuttled into mammalian cells by the cell-penetrating peptide penetratin. *Antimicrobial Agents and Chemotherapy* **56**, 5194-5201
80. Rerole, A. L., Gobbo, J., De Thonel, A., Schmitt, E., de Barros, J. P. P., Hammann, A., Lanneau, D., Fourmaux, E., Deminov, O., Micheau, O., Lagrost, L., Colas, P., Kroemer, G., and Garrido, C. (2011) Peptides and aptamers targeting HSP70: a novel approach for anticancer chemotherapy. *Cancer Research* **71**, 484-495
81. Kanzaki, H., Saitoh, H., Ito, A., Fujisawa, S., Kamoun, S., Katou, S., Yoshioka, H., and Terauchi, R. (2003) Cytosolic HSP90 and HSP70 are essential components of INF1-

mediated hypersensitive response and non-host resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana*. *Molecular Plant Pathology* **4**, 383-391

82. Su, P. H., and Li, H. M. (2008) Arabidopsis stromal 70-kD heat shock proteins are essential for plant development and important for thermotolerance of germinating seeds. *Plant Physiol.* **146**, 1231-1241

CHAPTER 6

PINE DIFFERENTIAL EXPRESSION RESPONSES TO NOCTILISIN AND WHOLE SIREX NOCTILIO VENOM

6.1 Introduction

Phenotypic effects of *Sirex noctilio* attack in *Pinus* species are well-documented. Specifically, more is known about the effects of *S. noctilio* venom than effects from the fungus, in part due to the focus of most modern research on this pathosystem (1,2). *S. noctilio* venom causes pine needles starch accumulation within 5-10 days after attack, wilting, needle chlorosis and abscission of older needles (3-5). Other recorded pine responses to *S. noctilio* venom include reduced radial stem growth, increased needle starch accumulation, and reduced needle pressure (6). Later pine responses attributed specifically to venom included increased amylase and peroxidase activity in needles and needle phloem element collapse (7).

Less well-documented are the responses in pines to *S. noctilio* venom at the level of gene expression. Both loblolly (*P. taeda*) and Monterey (*P. radiata*) pines have demonstrated measurable increases in transcript levels of pathogenesis-related protein (PR4), thaumatin-like protein (TLP) and, to a lesser degree, phenylalanine ammonium lyase 1 (PAL1)(Chapter 3, this work). Pathogen-related (PR) proteins are defense proteins induced in many plant species by various pathogens or pests, and they are often associated with disease resistance (8,9). PR4 proteins, which are typically localized in the vacuole, have been shown to harbor either chitinase or antifungal activity, or both (10,11). TLPs comprise another ubiquitous group of PR proteins

(classified as PR5 family). TLP gene transcripts have been shown to increase in response to pathogens in various forest trees species, including members of the genera *Castanea*, *Picea*, *Populus*, and *Pseudotsuga* (12-14). TLPs also respond to abiotic stimuli such as cold, drought, or salt stress (14). In contrast, two actin genes (ACT1, ACT2) are reasonably stable to venom exposure (2). Field-grown pines of several species have also been tested for gene-level venom responses via needle-based biomarker assays, but results so far have been equivocal (see Appendix to this dissertation).

Pine gene-level responses to *S. noctilio* venom vary by species. *P. radiata* is generally more sensitive to *S. noctilio* venom than *P. taeda*. Quantitative real-time PCR assays revealed species-level differences for induction of PR4 and TLP gene expression in response to venom. *P. radiata* showed a nearly 50-fold difference in up-regulation of PR4 and a nearly 100-fold difference in up-regulation of TLP relative to *P. taeda* (2).

Further, individual trees within several pine species can display widely variable traits because of the high genetic diversity in existing populations (15). Early researchers noted qualitative differences in the response to *S. noctilio* venom among *P. radiata* individuals (5-7,16,17). But while relatively insensitive trees were identified, and attacked trees were often found to survive, even relatively insensitive trees were found to be killed under sufficiently high rates of oviposition (17). Earlier researchers utilized in their bioassays tissue from trees previously rated as highly sensitive to *S. noctilio* venom (7,18), suspecting a genetic basis for the differential responses of individual *P. radiata* trees to *S. noctilio* venom (4,17). Modern studies have demonstrated numerous changes in gene expression underlying physiological responses to biotic (e.g., insect attack) and abiotic (e.g., drought) stress in plants (19,20). QRT-PCR analysis of PR4 and TLP biomarkers showed a 10-fold difference between venom-induced transcription

levels across two *P. radiata* genotypes (2). The levels of gene expression corresponded roughly to the strength and speed of the observable phenotypic changes induced by venom.

Many of these responses involve changes in gene transcription that can be followed using differential expression measurements made available through next-generation sequencing. It would be helpful to identify and characterize the full complement of genes responding to *S. noctilio* venom exposure. Additionally, it would provide a great deal of insight to distinguish the effects of whole venom from those associated with the primary causative agent of the wilt phenomenon, noctilisin. Isolating these effects from one another would simultaneously offer more specific assays for noctilisin activity and clues to its mechanism, while informing the search for other activities related to venom exposure in pines. Further, the timing of these expression events coupled with their magnitude would be a useful measure of significance of the specific expression event.

6.2 Materials and Methods

Two four year old *P. radiata* hedged seedlings, previously rated as venom-sensitive, were used. From each seedling, two cuttings were used per time point for each of three treatment types: whole venom, native noctilisin, or aglyconoctilisin, a glycan-free synthetic peptide previously shown to be inactive in qRT-PCR-based biomarker assays. Whole venom test solution was a 20 mg/ml water extraction from homogenized whole *S. noctilio* venom gland/sac combined apparatus. Noctilisin was HPLC-purified 97 μ M solution in water. Synthetic peptide used was 30 μ M aglyconoctilisin in water. Under these test conditions, a quantity of 12 cuttings per seedling was limiting; additional time points or treatments requiring further sampling of

seedlings may have endangered the seedlings. At four or 24 hours, cuttings were flash frozen in liquid nitrogen and stored at -80° C.

Frozen cuttings were ground by mortar and pestle, and RNA extracted as described previously (2). DNase-treated samples were supplied to the Georgia Genomics Facility at the University of Georgia (<http://dna.uga.edu/>) for library preparation by standard protocols

Twenty four samples (2 cuttings x 3 treatments x 2 time points x 2 genotypes) were run on an Illumina HiSeq 2000 instrument, multiplexing for 1 lane. Estimated per sample base coverage is approximately 12 fold; per sample read coverage is approximately 7,791,666 reads.

6.3 Results, Conclusions

At the time of writing the dissertation, samples prepared and submitted for RNA-Seq analysis were in a queue awaiting sequencing. As such, no conclusions have been reached yet.

References

1. Bordeaux, J. M., and Dean, J. F. D. (2012) Susceptibility and response of pines to *Sirex noctilio*. in *Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest* (Slippers, B., DeGroot, P., and Wingfield, M. J. eds.), Springer, New York. pp 31-50
2. Bordeaux, J. M., Lorenz, W. W., and Dean, J. F. D. (2012) Biomarker genes highlight intraspecific and interspecific variations in the responses of *Pinus taeda* L. and *Pinus radiata* D. Don to *Sirex noctilio* F. acid gland secretions. *Tree Physiol.* **32**, 1302-1312
3. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .I. Effects of symbiotic fungus *Amylostereum* sp. (Thelophoraceae). *Aust. J. Biol. Sci.* **22**, 915-924
4. Coutts, M. P. (1968) Rapid physiological change in *Pinus radiata* following attack by *Sirex noctilio* and its associated fungus, *Amylostereum* sp. *Aust. J. Sci.* **30**, 275-277
5. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .2. Effects of *S. noctilio* mucus. *Aust. J. Biol. Sci.* **22**, 1153-1161
6. Spradbery, J. P. (1973) A comparative study of the phytotoxic effects of siricid woodwasps on conifers. *Ann. Appl. Biol.* **75**, 309-320
7. Fong, L. K., and Crowden, R. K. (1973) Physiological effects of mucus from wood wasp, *Sirex noctilio* F., on foliage of *Pinus radiata* D. Don. *Aust. J. Biol. Sci.* **26**, 365-378
8. van Loon, L. C., Rep, M., and Pieterse, C. M. J. (2006) Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology* **44**, 135-162
9. (2006) *Multigenic and induced systemic resistance in plants*, Springer, 233 Spring Street, New York, Ny 10013, United States

10. Gu, Y. Q., Wildermuth, M. C., Chakravarthy, S., Loh, Y. T., Yang, C. M., He, X. H., Han, Y., and Martin, G. B. (2002) Tomato transcription factors Pti4, Pti5, and Pti6 activate defense responses when expressed in Arabidopsis. *Plant Cell* **14**, 817-831
11. Li, X., Xia, B., Jiang, Y., Wu, Q., Wang, C., He, L., Peng, F., and Wang, R. (2010) A new pathogenesis-related protein, LrPR4, from *Lycoris radiata*, and its antifungal activity against *Magnaporthe grisea*. *Molecular Biology Reports* **37**, 995-1001
12. Veluthakkal, R., and Dasgupta, M. G. (2010) Pathogenesis-related genes and proteins in forest tree species. *Trees-Struct. Funct.* **24**, 993-1006
13. Liu, J.-J., Zamani, A., and Ekramoddoullah, A. K. M. (2010) Expression profiling of a complex thaumatin-like protein family in western white pine. *Planta* **231**, 637-651
14. Liu, J. J., Sturrock, R., and Ekramoddoullah, A. K. M. (2010) The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function. *Plant Cell Reports* **29**, 419-436
15. Brown, G. R., Gill, G. P., Kuntz, R. J., Langley, C. H., and Neale, D. B. (2004) Nucleotide diversity and linkage disequilibrium in loblolly pine. *Proc. Natl. Acad. Sci. USA* **101**, 15255-15260
16. Coutts, M. P. (1970) The physiological effects of the mucus secretion of *Sirex noctilio* on *Pinus radiata*. *Aust. For. Res.* **4**, 23-26
17. Kile, G. A., Bowling, P. J., Dolezal, J. E., and Bird, T. (1974) The reaction of *Pinus radiata* twigs to the mucus of *Sirex noctilio* in relation to resistance to *Sirex* attack. *Aust. For. Res.* **6**, 25-34

18. Wong, L. K., and Crowden, R. K. (1976) Preliminary studies on mucus secretion of wood wasp, *Sirex noctilio* F .1. Physicochemical and biochemical properties. *Aust. J. Biol. Sci.* **29**, 21-32
19. Lorenz, W. W., Alba, R., Yu, Y.-S., Bordeaux, J. M., Simoes, M., and Dean, J. F. D. (2011) Microarray analysis and scale-free gene networks identify candidate regulators in drought-stressed roots of loblolly pine (*P. taeda* L.). *BMC Genomics* **12**
20. Wei, H., Persson, S., Mehta, T., Srinivasasainendra, V., Chen, L., Page, G., Somerville, C., and Loraine, A. (2006) Transcriptional coordination of the metabolic network in *Arabidopsis*. *Plant Physiol* **142**, 762 – 774

CHAPTER 7

CONCLUSIONS

7.1 Noctilisin Initiates the *Sirex noctilio* Pine Wilt Pathology

Sirex noctilio, a non-native and known invasive woodwasp from Europe now present in North America, can kill healthy pines by introducing venom and a fungal pathogen into tree tissues during oviposition. The venom alone is responsible for initiating the pathology causing trees eventually to succumb, and is associated with needle wilt, phloem collapse, and the shutdown of photosynthate transport as evidenced by starch accumulation in needles and carbohydrate depletion in stems. Trees in this weakened state are rendered less able to fend off the otherwise mildly pathogenic white rot basidiomycete *Amylostereum areolatum* vectored by the woodwasp at oviposition.

The activity in the venom was tracked through fractionation by means of two bioassay types: phenotypic and expression-based. Phenotype assays are not feasible for mature pines, but model systems based upon whole seedlings or explants removed from hedged seedlings, especially tissue from particularly venom-sensitive *Pinus radiata*, were used effectively to track visible symptoms. Expression-based bioassays were developed using a *P. taeda* cDNA microarray to identify marker genes upregulated in *P. taeda* stems exposed to *S. noctilio* venom at both 24 and 48 hours. Pathogenesis-related protein 4 (PR4) and thaumatin-like protein (TLP) genes were used to model pine response to venom, while an actin gene (ACT1) was identified as stable to venom exposure. Biomarker assays must correlate with the wilt phenotype and ideally

respond quickly and consistently to venom exposure. Both PR4 and TLP were well-suited to this purpose. Pine response to *S. noctilio* venom was recorded in whole seedlings and cultured cells (*P. taeda*, cell culture data not shown) as early as four hours, and in *P. radiata* stems from hedge seedlings. Gradients of response sensitivity were recorded both across species (*P. radiata* more sensitive than *P. taeda*) as well as within species (among genotypes). Literature suggested that the bioactive factor from venom likely was a small, highly water-soluble polypeptide. Our proteolytic experiments on venom appeared to augment this evidence.

As a polypeptide, the active factor must necessarily be coded for by messenger RNA. As such, simultaneously with venom fractionation, the venom tissue from *S. noctilio* females was sampled, total RNA was extracted, and high-throughput, massively parallel sequencing was performed. The dataset was assembled into the venom transcriptome, the first known for a tissue from a member of the Symphyta, a basal suborder of the hymenopteran insects. As it happened, the most abundant transcript in venom was a species with no homology to annotated sequences in public databases, and this transcript contained the sequence for the active factor. Additionally, the most abundant annotated transcript shared homology with a laccase-like multicopper oxidase (LMCO) found in *Anopheles gambiae*. We demonstrated laccase activity via zymogram, and later proteomic analysis also revealed fragments of a LMCO in abundance from venom. The significance of the LMCO for woodwasp survival is obscure, but the insect's heavy investment in this enzyme strongly suggests its importance.

As the venom active factor was remarkably heat-stable, during fractionation venom tissue was macerated in water, boiled, and centrifuged, the supernatant passed through a 10 kDa centrifugal concentrator, and the pass-through separated via RP-HPLC. Using the expression-based bioassay on cut stems from sensitive hedged *P. radiata* seedlings, the active factor causing

wilt and upregulation of PR4 and TLP biomarker genes was isolated. Its identity, purity, and structure were established utilizing Edman degradation for primary sequence, then confirmed by both transcriptome data and NMR. MALDI-TOF established the molecular weight of the pure substance as 1850 Da, higher than expected based on the primary sequence data. NMR then established the presence of modified GalNAc moieties O-linked in an alpha configuration to serinyl and threonyl residues, as well as a 4-hydroxyl modification on prolinyl residue 6. Phosphorus NMR confirmed the presence of identical ethanolamine modifications to GalNAc C6. Tandem mass spectrometry and collision-induced dissociation confirmed the presence of fragments consistent with NMR results. A synthetic peptide with the hydroxyprolinyl residue but absent the two glycans failed to show activity in the bioassay, establishing the glycan-dependence of the interaction. Finally, UV-VIS at 190 nm established a means to quantify the active factor by measuring the peptide backbone alone.

Noctilisin has been identified as a post-translationally modified, eleven-amino acid glycopeptide with primary sequence SEGPROGTKRP. Its mode of action appears to be linked to the O-glycosylation of its serine and threonine residues. Learning which genes respond specifically to noctilisin among the thousands of components introduced into pine during woodwasp oviposition is now possible using this pure material. We have exposed stems from two sensitive genotypes of *P. radiata* to whole venom (water maceration, 20 mg/ml), noctilisin (97 μ M) or a synthetic peptide identical to noctilisin but without the O-linked glycans (30 μ M), a compound demonstrated to have no effect in expression and phenotype-based bioassays. These assays were performed in duplicate and sampled at two time points: 4 and 24h. Extracts of total RNA from these samples were sent for massively parallel sequencing via Illumina technology (RNA-Seq). The samples from this analysis are awaiting sequencing at the time of this

manuscript's production, but they are expected to offer a clear indication of gene cohorts responding exclusively to noctilisin or to whole venom. More specific noctilisin response biomarkers may result from this analysis, which can then be validated by qRT-PCR. Additionally, surveys of the genes responding to each solution will offer clues as to the mechanism of action, both at the site of oviposition (from whole venom) and distally (from noctilisin, the translocated active factor). Weighted correlation gene network analysis (WCGNA) is one of many tools that can be applied to these data (1). Such analysis can yield hub genes, those genes which underlie the expression of many others. In this way much more specific sources for expression changes induced by the initial perturbant, may be revealed, specifically by genotype and exposure time.

Sirex noctilio-caused wilt is only one of a suite of pine responses to introduction of venom during oviposition. It is expected that there are site-specific plant responses to woodwasp venom, judging from the obvious phenotype at the site of oviposition (resinosis, accumulation of phenolics, tissue death). Many more reductive experiments are necessary to completely isolate site-specific venom effects from those of the symbiotic fungus, *Amylostereum areolatum*. Fortunately, early researchers have laid the groundwork for such analysis. Building on this knowledge with the elucidation of suites of genes responding specifically to whole venom versus noctilisin should offer many clues. For instance, were we to learn that genes normally responsive to drought, heat, or nitrogen deficiency are induced by whole venom, specific hypothetical mechanisms may be indicated which may then be tested. We already know that an active phenoloxidase is present in whole venom and that another LMCO is secreted by the fungus. Phenolic compounds are known to accumulate near the site of oviposition. Perhaps gene network analysis will indicate that phenolics are induced by an LMCO in venom, calling into question the

previous hypothesis that phenolics are produced in pine solely through exposure to fungal LMCOs. Differential expression of phenylpropanoid gene transcripts may indicate this pathway is altered by the presence of venom. This is one of many possible hypothesis-generating outcomes expected from the pine RNA-Seq experiment.

7.2 Venom: Transcriptome, Proteome, Glycosylation

Characterization of noctilisin, the initial causative agent in a major worldwide plant pathosystem, is an important step toward understanding the mechanism by which it exerts its effects in trees. Moreover, screens for future breeding stock for pines resistant to *S. noctilio* venom now are feasible.

Hymenopteran venoms share in common a diversity of bioactive compounds. Among these, peptide components can comprise as much as 70% of venom dry weight. Melittin is a 26-a.a. peptide comprising nearly 50% of honeybee (*Apis mellifera*) venom. It assumes a bent alpha-helical configuration, and acts as a chaotropic agent on cell membranes. Apamin (18 a.a.) and mast cell-degranulating peptide (MCD, 22 a.a.), both also from honeybee, form ring structures in solution; apamin acts on the central nervous system of mammals, while MCD is a cytolytic peptide. Mastoparans (14 a.a.), linear, polycationic peptides, are found in the venom of social wasps and stimulate exocytosis in conjunction with, or absent interactions with, G-protein receptors. Crabrolin (13 a.a.), from *Vespa crabro* venom, is similar in activity to mastoparans but less potent, and assumes an alpha-helical configuration (2). None of these small, bioactive hymenopteran venom peptides are known to assert their effects in a glycosylated form. A larger hymenopteran venom protein, aspartylglucosaminidase isolated from the parasitoid wasp *Asobara tabida*, does assert its effects in glycosylated form (3). Formaecin I (16 a.a.) is a peptide

isolated from the ant *Myrmecia gulosa* with a single GalNAc O-linked to threonine. Both the insect-derived glycopeptide and a synthetic peptide consistently showed more potent bioactivity than a synthetic non-glycosylated form (4), but a program to produce equivalent activity in non-glycosylated alternative forms of the peptide has been successful (5). We have added to the roster of diverse hymenopteran bioactive compounds by isolating for the first time, to our knowledge, a pine response-inducing glycopeptide from the venom of a symphytan (basal suborder of Hymenoptera including woodwasps and sawflies).

Having isolated and structurally characterized the principal molecule inducing wilt pathology in trees attacked by *S. noctilio* woodwasps, the viability of several areas of future research are evident. As the molecule has been identified as a glycopeptide, the range of possible cell structures interacting with the molecule is limited by its size class. It may now be learned whether or not the molecule penetrates the plant cell membrane, or exerts its activity on the cell surface. Should it pass the membrane, it may act as a signal inducing a particular pathway inducing cell death, or it may be small enough to reach the nucleus where it could act as a transcription factor.

Purification of native noctilisin from woodwasp venom is at present much simpler and more cost-effective than producing it in a heterologous expression system, provided an ample supply of female insects is available. We also know that *P. taeda* suspension cultured cells are responsive to *S. noctilio* venom at the level of transcription as early as four hours post-exposure (data not shown).

Further studies will benefit from a means of visualizing the peptide's pathway through tissue to localize its activity to a cell component. It may be possible to perform microscopic visualization experiments on suspension-cultured pine cells to track noctilisin via its labeled

identical GalNAc moieties. By the same means, immunocytochemistry and microscopy may be combined to track the progress of noctilisin through the stem and needle tissue of whole seedlings. The presence of the GalNAc moiety should be particularly effective here: it is equivalent to the Tn antigen, for which commercial antibody kits already are available. The viability of labeling the modified Tn antigen on noctilisin with these fluorophores has yet to be tested, however, and it is not known whether interference from the ethanolamine modification on GalNAc C6 may prevent effective labeling. If commercial labeling is not effective, it is also possible to raise antibodies to the now-purified glycopeptide.

One further visualization technique, radioisotope labeling, would be particularly effective in understanding mechanism: a single labeled atom on the glycopeptide could be used without fear of any possible steric interference by a large attached fluorophore. This kind of system could be expensive, and it may require heterologous expression systems. Nonetheless, the mechanism for noctilisin action on or in plant cells is now within reach.

The *S. noctilio* venom transcriptome offered the first insights into the protein composition of a non-apocritan hymenopteran venom, a venom which has demonstrated bioactivity involved in the pathosystem against a major forest tree species. Proteomic data generated recently (Appendix B) has validated some of the findings from the venom transcriptome RNA-Seq data. From these peptide fragments we may focus clearly on correct reading frames for future analyses. We also demonstrated the presence of proteins coded for by the transcriptome, and correlated some of these with known activities (laccase, amylase, and protease) or suspected activities (N-acetylgalactosaminyl transferase) present in *S. noctilio* venom. Interestingly, several of the major findings within the venom proteome support the presence of glycan-containing

moieties as yet uncharacterized: a fucosyltransferase and a trehalase are among the most abundant proteins found in venom.

Glycosylation of proteins and peptides is extraordinarily widespread in nature; by one estimate, 50% of all known eukaryotic proteins are expected to be characterized as glycoproteins (6). Glycans may be N-linked, O-linked, C-linked, or GPI anchor-associated (7). Cell surfaces are covered abundantly with carbohydrates, protecting the cell but also mediating information exchange (8). Glycosylation is involved in protein folding, sorting, and targeting (9); adhesion and recognition between cells (10); the binding of pathogens (11); and it plays a direct role in some plant pathogen-host interactions (12).

Aglyconoctilisins (SEGPROGTKRP) were synthesized to probe the functional significance of glycosylation for noctilisin. The glycan-free peptide, run as a control, produced none of the effects either of native noctilisin or of whole venom. This suggests that the activity, and possibly the stability, solubility, or mobility, of the insect-derived peptide is enhanced by the O-glycosylation. Given the heat-stability of the insect peptide along with its ability to travel intact to distant sites within the plant, it is unsurprising that glycosylation plays a critical role in its activity. Glycosylation is well-characterized as a means of protecting cell surfaces and as a modification whereby peptide moieties can avoid proteolysis and reach their biological targets (13,14). A similarly heat-stable extracellular glycopeptide causing wilt in alfalfa was isolated from *Corynebacterium insidiosum*, but this toxin was several orders of magnitude larger than noctilisin (15,16).

At least 48 transcripts found in the *S. noctilio* venom transcriptome gave BLAST returns annotating them as galactosyltransferases. Of these, twenty transcripts were identified having abundance of at least one transcript per million (TPM), and all carried E-values $< 10^{-100}$. Among

these, 70% contained a detectable secretion signal, including the most abundant transcript with this annotation. This seems to support O-glycosylation of noctilisin post-translationally (Table 7.1). Further, 16 of these transcripts code for N-acetylgalactosaminyltransferases (Tn-antigen synthesis). As noted in Appendix B, the first entry in Table 7.1 has been positively identified in the proteome of venom as one of the most abundant proteins in the venom samples analyzed (Table 7.2). The remaining four transcripts were annotated as glycosyltransferases responsible for transferring a galactose to the Tn antigen, generating the core 1 O-glycan (T-antigen synthesis). T-antigen is a precursor for extended carbohydrate moieties, many of which are associated with pathological states in animals (17). Extended glycosylation certainly appears to be a feature of the bulk venom, given its viscous, mucus-like physical properties.

7.3 Enhancement of Future Pine Breeding Populations

Understanding which pine genes respond to various components of the venom is a first step in identifying trees that may harbor inherent resistance to the effects of venom. Using purified noctilisin in needle bioassays may offer a non-destructive means to screen existing adult trees for resistance to venom effects. It remains obscure how wilt correlates with resistance or susceptibility to venom, but early studies appear to correlate wilt with a disadvantage for the trees (18,19). Because one of the major symptoms in pines exposed to *S. noctilio* venom is the cessation of photosynthate transport and subsequent depletion of stem carbohydrate, such a disruption in transport of metabolic fuel would not appear conducive to resistance. On the other hand, another study felt that early wilt was a tree response that mobilized plant defense against the venom, and that trees wilting early and rapidly were better able to recover (20). This point

must be firmly settled, both by species and intraspecifically, to correctly identify pine populations with actual specific resistance to attack by *S. noctilio*.

7.4 Concluding Remarks

Noctilisin is a small venom glycopeptide altering expression and metabolism in a woody plant species, the first hymenopteran venom exhibiting such activity. The identity of the glycopeptide initiating tree responses, enhanced by the discovery of modified Tn antigens O-linked to its primary structure, will likely be key to identifying the mechanism of noctilisin action in pine. A functional bioassay for venom-induced expression responses in pine is now available. The *S. noctilio*-vectored pathosystem is now tractable to a variety of cell biological, molecular, and biochemical methods of analysis. The molecular toolkit for studying this system will be further expanded by the results of the RNA-Seq comparison of pine responses to venom fractions.

This study confirmed the presence, identity, and activity of an LMCO in venom, augmenting less-convincing previous reports in the literature. Its presence and abundance is intriguing, and the significance of this enzyme to the pathosystem remains to be explored. Several of the most abundant transcripts identified in *S. noctilio* venom are new to science, having no homologs in public databases. The *S. noctilio* venom transcriptome is now publicly available, and it expands the scope of comparative venomomics to include a basal suborder of the important insect order Hymenoptera. Proteomic analysis supports previous reports of amylase in venom, as well as the presence of enzymes known to be necessary for post-translational modifications to noctilisin as well as other glycosylation of polypeptides.

Conventional tree-improvement efforts will be supported by molecular methods for screening breeding populations of pines for resistance to a worldwide killer of trees, *Sirex noctilio*. Future efforts to control this and other phytophagous insects will be enhanced through the insights provided in this study.

Table 7.1: *S. noctilio* venom transcripts coding for galactosyltransferases. Information generated from Illumina sequencing of the entire *S. noctilio* venom tissue (Bordeaux et al., submitted). Transcripts with TPM < 1 are excluded. Annotations in bold are transcripts responsible for glycosylation of the Tn antigen, creating the core 1 O-glycan T-antigen.

Sirex DB ID no.	Predicted secretion signal	TPM	Accession no.	Annotation	Query match (a.a.)	% Identity	% Conserved	Expect value
comp10716_c6_seq1	yes	66.0	ref XP_003494505.1	putative polypeptide N-acetylgalactosaminyltransferase 9-like isoform 1 [<i>B. impatiens</i>]	602	86.0%	91.4%	0
comp11176_c0_seq1	--	6.8	ref XP_003707310.1	N-acetylgalactosaminyltransferase 6-like [<i>M. rotundata</i>]	278	81.3%	91.4%	0
comp11102_c1_seq3	yes	5.8	ref XP_003708165.1	beta-1,4-N-acetylgalactosaminyltransferase bre-4-like [<i>M. rotundata</i>]	395	72.9%	84.3%	0
comp10802_c2_seq8	yes	5.7	ref XP_003708078.1	polypeptide N-acetylgalactosaminyltransferase 5-like [<i>M. rotundata</i>]	608	85.0%	90.8%	0
comp10166_c2_seq8	yes	4.5	gb EFN86351.1	glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 [<i>H. saltator</i>]	296	81.1%	87.8%	1.00E-179
comp8011_c0_seq1	yes	4.5	ref XP_624054.3	beta-1,4-galactosyltransferase 7 [<i>A. mellifera</i>]	264	79.5%	90.5%	1.00E-156
comp10802_c2_seq7	yes	4.1	ref XP_003708078.1	polypeptide N-acetylgalactosaminyltransferase 5-like [<i>M. rotundata</i>]	608	85.0%	90.8%	0
comp10189_c0_seq4	--	3.9	gb EFN62008.1	N-acetylgalactosaminyltransferase 7 [<i>C. floridanus</i>]	581	85.9%	92.4%	0
comp10777_c0_seq2	yes	3.4	ref XP_003402286.1	polypeptide N-acetylgalactosaminyltransferase 35A-like [<i>B. terrestris</i>]	646	74.1%	86.5%	0
comp10802_c2_seq6	yes	2.9	ref XP_003708078.1	polypeptide N-acetylgalactosaminyltransferase 5-like [<i>M. rotundata</i>]	608	85.0%	90.8%	0
comp10166_c2_seq7	yes	2.6	gb EGI57884.1	glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 [<i>A. echinator</i>]	349	79.4%	87.7%	0
comp10802_c2_seq4	yes	2.4	ref XP_003708078.1	polypeptide N-acetylgalactosaminyltransferase 5-like [<i>M. rotundata</i>]	608	85.0%	90.8%	0
comp9149_c1_seq1	--	2.3	ref XP_003692629.1	beta-1,3-galactosyltransferase 6-like [<i>A. florea</i>]	200	71.0%	85.0%	1.00E-115
comp10189_c0_seq3	--	2.2	gb EFN62008.1	N-acetylgalactosaminyltransferase 7 [<i>C. floridanus</i>]	581	85.9%	92.4%	0
comp10802_c2_seq5	yes	2.1	ref XP_003708078.1	polypeptide N-acetylgalactosaminyltransferase 5-like [<i>M. rotundata</i>]	608	85.0%	90.8%	0
comp10802_c2_seq3	yes	1.7	ref XP_003708078.1	polypeptide N-acetylgalactosaminyltransferase 5-like [<i>M. rotundata</i>]	608	85.0%	90.8%	0
comp11102_c1_seq1	yes	1.6	ref XP_003708165.1	beta-1,4-N-acetylgalactosaminyltransferase bre-4-like [<i>M. rotundata</i>]	395	72.9%	84.3%	0
comp10802_c2_seq2	yes	1.2	ref XP_003708078.1	polypeptide N-acetylgalactosaminyltransferase 5-like [<i>M. rotundata</i>]	608	85.0%	90.8%	0
comp9760_c0_seq1	--	1.1	gb EFN85545.1	beta-1,3-galactosyltransferase 1 [<i>H. saltator</i>]	377	69.5%	81.4%	1E-177
comp10189_c0_seq2	--	1.0	gb EFN62008.1	N-acetylgalactosaminyltransferase 7 [<i>C. floridanus</i>]	581	85.9%	92.4%	0

Table 7.2: Peptide fragments obtained for an apparent peptidyl-GalNAc transferase in *S. noctilio* woodwasp venom.

Sequence Id	Significance	Frame	TPM
comp10716_c6_seq1	putative polypeptide N-acetylglycosaminyltransferase [<i>B. impatiens</i>]	+3	66
Fragments	Found in ORF?		
RHQQAEPVWSPTMAGGLFSIDRA	yes		
RIGHETGDYGDVSERK	yes		
KALLHCHPFMPNVFLVFFSFISTRYNNANTTTEFILHSKI	yes, first A miscalled as a stop		
KRKFHMSDDARR	yes, first F miscalled as a stop		
RMREIIGYTNLYNYTRI	yes, second I miscalled as a stop		

References

1. Langfelder, P., and Horvath, S. (2008) WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* **9**
2. Palma, M. S. (2006) Insect venom peptides. in *Handbook of Biologically Active Peptides* (Kastin, A. J. ed.), Academic Press, New York. pp 389-396
3. Vinchon, S., Moreau, S. J. M., Drezen, J. M., Prevost, G., and Cherqui, A. (2010) Molecular and biochemical analysis of an aspartylglucosaminidase from the venom of the parasitoid wasp *Asobara tabida* (Hymenoptera: Braconidae). *Insect Biochem. Mol. Biol.* **40**, 38-48
4. Mackintosh, J. A., Veal, D. A., Beattie, A. J., and Gooley, A. A. (1998) Isolation from an ant *Myrmecia gulosa* of two inducible O-glycosylated proline-rich antibacterial peptides. *J. Biol. Chem.* **273**, 6139-6143
5. Kaur, K. J., Pandey, S., and Salunke, D. M. (2007) Design of a functionally equivalent nonglycosylated analog of the glycopeptide antibiotic formaecin I. *Protein Sci.* **16**, 309-315
6. Apweiler, R., Hermjakob, H., and Sharon, N. (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim. Biophys. Acta Gen. Subj.* **1473**, 4-8
7. Wei, X., and Li, L. (2009) Comparative glycoproteomics: approaches and applications. *Brief. Funct. Genomics Proteomics* **8**, 104-113
8. Hang, H. C., and Bertozzi, C. R. (2005) The chemistry and biology of mucin-type O-linked glycosylation. *Bioorganic Med. Chem.* **13**, 5021-5034

9. Helenius, A., Aebi, and Markus. (2001) Intracellular functions of N-Linked glycans. *Science* **291**, 2364-2369
10. Zhang, L. P., and Ten Hagen, K. G. (2011) The cellular microenvironment and cell adhesion: a role for O-glycosylation. *Biochem. Soc. Trans.* **39**, 378-382
11. Dallas, D. C., Martin, W. F., Hua, S., and German, J. B. (2013) Automated glycopeptide analysis-review of current state and future directions. *Brief. Bioinformatics* **14**, 361-374
12. Fernández-Álvarez, A., Elías-Villalobos, A., and Ibeas, J. I. (2010) Protein glycosylation in the phytopathogen *Ustilago maydis*: from core oligosaccharide synthesis to the ER glycoprotein quality control system, a genomic analysis. *Fungal Genet. Biol.* **47**, 727-735
13. Gill, D. J., Clausen, H., and Bard, F. (2011) Location, location, location: new insights into O-GaINAc protein glycosylation. *Trends in Cell Biol.* **21**, 149-158
14. Garner, B., Merry, A. H., Royle, L., Harvey, D. J., Rudd, P. M., and Thillet, J. (2001) Structural elucidation of the N- and O-glycans of human apolipoprotein(a): role of O-glycans in conferring protease resistance. *J. Biol. Chem.* **276**, 22200-22208
15. Ries, S. M., and Strobel, G. A. (1972) A phytotoxic glycopeptide from cultures of *Corynebacterium insidiosum*. *Plant Physiol.* **49**, 676-684
16. van Alfen, N. K., and Turner, N. C. (1975) Changes in alfalfa stem conductance induced by *Corynebacterium insidiosum* toxin. *Plant Physiol.* **55**, 559-561
17. Ju, T., Otto, V. I., and Cummings, R. D. (2011) The Tn antigen—structural simplicity and biological complexity. *Angewandte Chemie International Edition* **50**, 1770-1791
18. Coutts, M. P. (1969) Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .2. Effects of *S. noctilio* mucus. *Aust. J. Biol. Sci.* **22**, 1153-1161

19. Bowling, J. P., and Dolezal, J. E. (1970) Initial note on the development of a mucus test to determine resistance of *Pinus radiata* to attack by *Sirex noctilio*. *Aust. For. Res.* **5**, 57-62
20. Fong, L. K., and Crowden, R. K. (1973) Physiological effects of mucus from wood wasp, *Sirex noctilio* F., on foliage of *Pinus radiata* D. Don. *Aust. J. Biol. Sci.* **26**, 365-378

APPENDIX A

HIGH VARIABILITY IN BIOMARKER GENE RESPONSES TO SIREX NOCTILIO
VENOM IN FIELD-GROWN PINES⁵

⁵ Bordeaux, J.M., D.R. Coyle, J.F.D. Dean, and K.J.K. Gandhi, 2013, Proceedings, 32nd Southern Forest Tree Improvement Conference, 86-93. Reprinted here with permission of the publisher.

A.1 Introduction

Sirex noctilio is an exotic forest pest in North America and is capable of causing significant economic damage in plantation and naturally-grown pines (*Pinus* spp.) (1). Hazard maps for *S. noctilio* are essential to predicting high-risk areas for establishment, and should account for such factors as stand density, edaphic effects, and host preference. Patterns in *S. noctilio* woodwasp host preference for southeastern pine species have previously been documented; the woodwasp preferred Virginia (*P. virginiana* Mill.) and white (*P. strobus* L.) pines to four other southeastern U.S. *Pinus* species in bolt assays (2). Though useful, these data are not sufficient to estimate host tree susceptibility. A susceptibility assay for trees in the field would have utility for predictions of *S. noctilio* establishment.

Early observations of *S. noctilio* attack on pines noted that needles showed a characteristic wilt phenotype ('flagging'), which on a cellular level was correlated with collapsed phloem cells (3). We were able to reproduce this phenotype in the laboratory (Fig. A.1). Previously, we demonstrated that laboratory-grown seedlings and cuttings from potted trees can be used to gauge tree susceptibility to *S. noctilio* venom by measuring gene expression changes in two biomarker genes (PR4 and TLP) (4). Biomarker gene response was detectable well before phloem collapse and wilt in needles. The assay has shown utility for measuring gene responses in several species of pine exposed to *S. noctilio* venom.

The purpose of the present study was to assess whether this biomarker assay would be similarly useful for detecting responses in a variety of field-grown *Pinus* species. We hypothesized that changes in biomarker gene expression in response to *S. noctilio* venom would be similar in field-grown pines of various species compared with seedlings grown under controlled conditions. We further hypothesized that these responses could be correlated with

susceptibility or resistance to *S. noctilio* attack in a species-specific manner. If successful, such an assay would enable us to more efficiently study insect preference and host tree responses to *S. noctilio* under field conditions, and should permit development of better predictive models and incursion risk maps prior to woodwasp establishment in southeastern U.S.

A.2 Materials and Methods

A.2.1 Gene Expression Assays

A biomarker assay of pine gene responses to *S. noctilio* venom using quantitative real-time polymerase chain reaction (qRT-PCR) was previously described for use in laboratory-grown loblolly (*P. taeda*) and Monterey (*P. radiata*) tissues (4). The assay measures changes in transcription levels for two defense-response genes in pine (PR4, pathogenesis-related protein; TLP, thaumatin-like protein) in comparison to several housekeeping (control) genes. For this study, actin (ACT1, ACT2), ubiquitin-conjugating factor (UBCF), and glyceraldehyde phosphate dehydrogenase (GAPDH) were used as control genes.

It has been widely recognized that pine gene sequences are highly conserved between species and as a consequence oligonucleotide primers developed for one species are often directly transferrable to other pine species (5-7). To test whether the primer pairs for the biomarker and control genes listed above would amplify genes from species to be tested, needles were collected from field-grown loblolly, longleaf (*P. palustris* Mill.), Scots (*P. sylvestris* L.), shortleaf (*P. echinata* Mill.), table mountain (*Pinus pungens* Lamb.), Virginia, and white pines. One to three needle fascicle bundles from a mature tree yielded sufficient RNA to perform reverse-transcriptase PCR (RT-PCR) assays in replicate. Needle sampling was minimally invasive and deemed unlikely to affect gene expression in needles elsewhere in the tree. RT-PCR

amplification produced products (amplimers) using RNA from each test species as template, and all products were the same size (Fig. A.2). This fact strongly suggested acceptable conservation of sequence between genes within these species of *Pinus*.

A.2.2 Site selection and sampling plan

Trees were sampled from a common garden (The Pinetum at Thompson Mills Forest) near Braselton, Georgia (34°07'22.82"N, 83°47'48.95"W). The Pinetum contains examples of all conifer species native to Georgia. Sampling took place in February 2012 with a complete replicate collected four weeks later (March 2012). Six pine species native to Georgia -- loblolly, longleaf, shortleaf, table mountain, Virginia, and white – as well as a European species that is a natural host to *S. noctilio* (Scots pine) were sampled for this study. Samples were taken from six trees of each species (N=6), except for table mountain and Scots pines (N=3). Four samples (fascicle bundles) were taken from each sampled tree, two of which were used for venom treatment, and two for water treatment (control). After exposure to venom or water for 24 h, needles were flash-frozen in liquid nitrogen, and RNA was extracted as previously described (4,8). Using protocols from the same studies, cDNA was synthesized from RNA and qRT-PCR was performed.

A.3 Results

Relative expression data for the biomarkers in venom- versus water-treated needles showed high variability between trees within species as well as between species (for example, between *P. strobus* vs. *P. palustris*). There was also high variability between the two biomarkers with respect to species response (Figs. A.3A, PR4; Fig. A.3B, TLP). Presenting data in terms of fold-change (Figs. A.3C, A.3D) was useful for identifying general trends in the responses of

some species, but did not provide for a reliable estimate of biomarker gene expression responses within a given species due to the high variability of response within species (see for example *P. sylvestris* and *P. palustris*). Variations between biomarkers in different species were especially pronounced (Figs. A.3C, A.3D).

Comparing biomarker gene responses in two southeastern U.S. pine species to Scots pine, a natural host of *S. noctilio*, in the North American species the two biomarkers showed a similar magnitude of response, but did not agree in direction. In *P. palustris*, for example, PR4 expression changed very little in response to venom, whereas TLP was strongly up-regulated by *S. noctilio* venom exposure (Fig. A.4). These results were not consistent with previous results using seedlings grown under controlled conditions, in which case expression was up-regulated for both biomarkers following venom exposure. Comparisons of biomarker expression changes in needles collected on different dates (Figs. A.4A, A.4B versus Figs. A.4C, A.4D) were somewhat more consistent, but any attempt to generalize from these data was confounded by high variability within species and between trials.

One observation of note from these experiments was that up-regulation of the biomarker genes was consistently stronger in Scots pine species compared to either of the southeastern native pines (Fig. A.5). Scots pine assays were particularly variable, which might be related to the small sample size (N=3 trees). While it was not possible to generalize broadly from these data, the magnitude of difference in biomarker expression (especially for TLP) in Scots pine relative to loblolly or longleaf pine was profound. Our previous studies showed that elevated levels of biomarker gene expressions was strongly correlated with the degree of wilt symptoms in needles (4).

A.4 Discussion

As sessile organisms that must cope with highly variable environmental conditions, plants monitor their growth conditions closely and respond quickly to changes by varying the expression levels of many different genes (9). Thus, it is not surprising that gene expression patterns observed when plants are grown under controlled conditions in a laboratory differ significantly from the expression patterns in field grown plants. Among the more responsive genes in this regard are those involved with plant defense responses (10). Such variations in gene expression are problematic for studies within species and even within clonal or isogenic lines, not to mention between the highly heterozygous genotypes that are typical of pine populations or between species. Inherent variability in expression of some genes may be dampened in such situations by using trees grown in a common garden, but various microclimatic conditions, including differential lighting, soil chemistry and drainage, and water supply, will still lead to variations between samples. Despite these issues, this study clearly demonstrated the utility of the PCR primer pairs developed on the basis of sequence information from loblolly pine for detecting and quantifying cognate transcripts in a wide variety of other pine species. Also, while the high degree of variability in gene expression from sample to sample indicate that these biomarker genes would be unreliable for detecting and assessing the degree of *S. noctilio* attack under field conditions, general trends across all experiments indicate that woodwasp venom tends to lead to increased expression of these genes to varying extents in the species tested.

Discoordinate expression of the two biomarker genes, PR4 and TLP, was not seen previously in venom-treated loblolly or Monterey pines grown under laboratory conditions, but was seen in several instances in this study. Thus, we observed that expression of the two biomarker genes differed not only in magnitude of relative expression (Figs. A.3C, A.3D) but in

direction, as well (Fig. A.4). Clearly field-grown trees offer a special challenge for the selection of useful biomarker genes, and experiments to identify potential biomarkers useful under these conditions will likely need to sample venom-treated field-grown trees.

One additional important observation from this study was that Scots pine, a European species that co-evolved with *S. noctilio*, consistently demonstrated stronger induction of biomarker gene expression in response to venom compared to all of the North American species we tested. Strong up-regulation of defense genes correlates with increased resistance in many plant-pest systems; however, it will take significantly more effort to establish whether or not this is the case for the *Sirex-Pinus* pathosystem. The degree of biomarker induction seen here indicates that Scots pine recognizes *S. noctilio* venom quickly and responds strongly. Further work with this species pairing could identify breeding targets for efforts to develop pines that are more resistant to this pest.

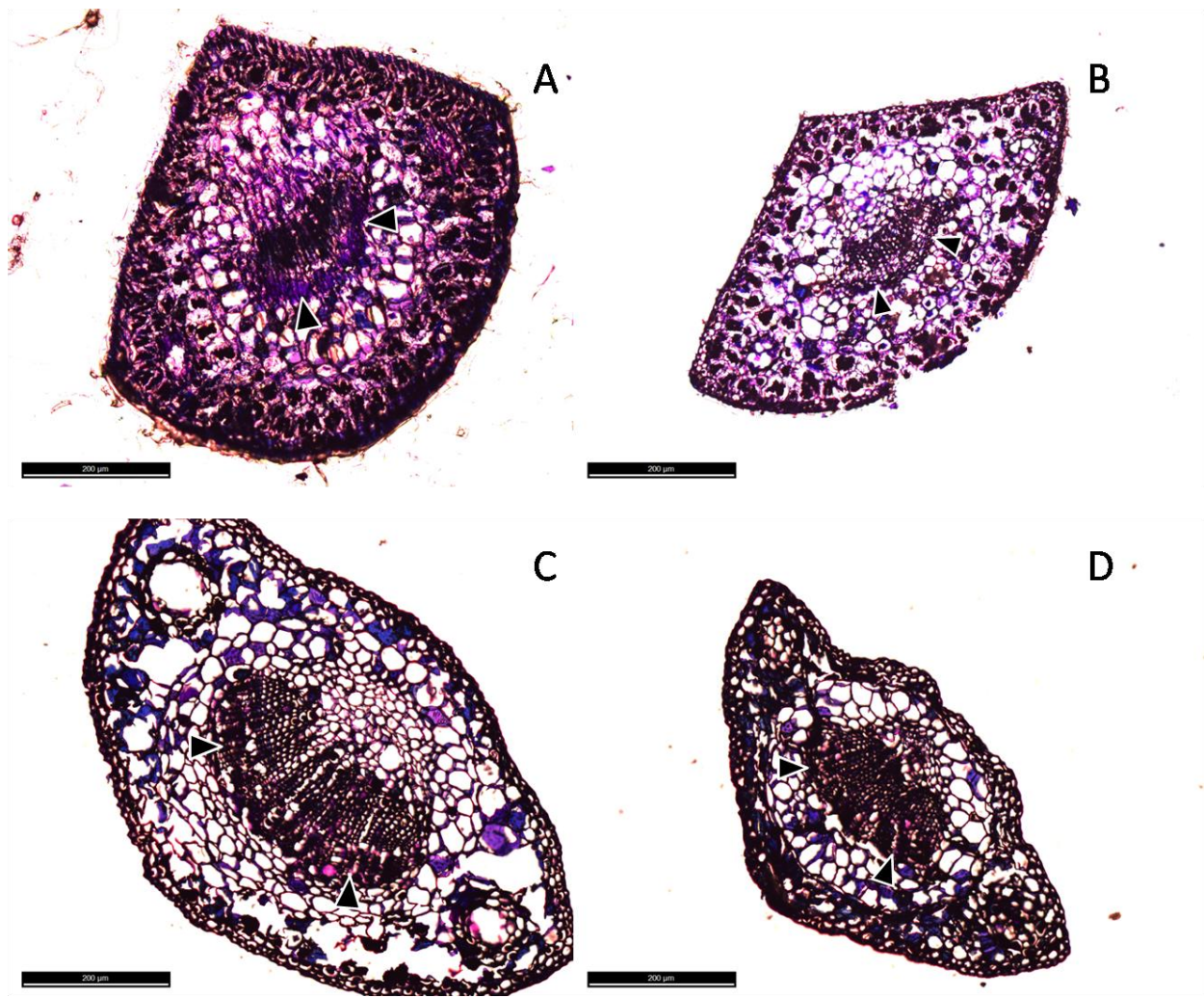


Figure A.1: Cross-sections of Monterey pine (*P. radiata*) needles exposed to fresh *S. noctilio* venom, 10X. Treated needles were fixed in 4% formaldehyde and sections were stained with toluidine blue. Needles were treated as follows: Panel A, 24 hrs water; B, 24 hrs fresh venom; C, 48 hrs water; D, 48 hrs fresh venom. The predominant cellular phenotype caused by venom treatment was collapse of phloem cells (arrows) as described by Fong and Crowden (3)

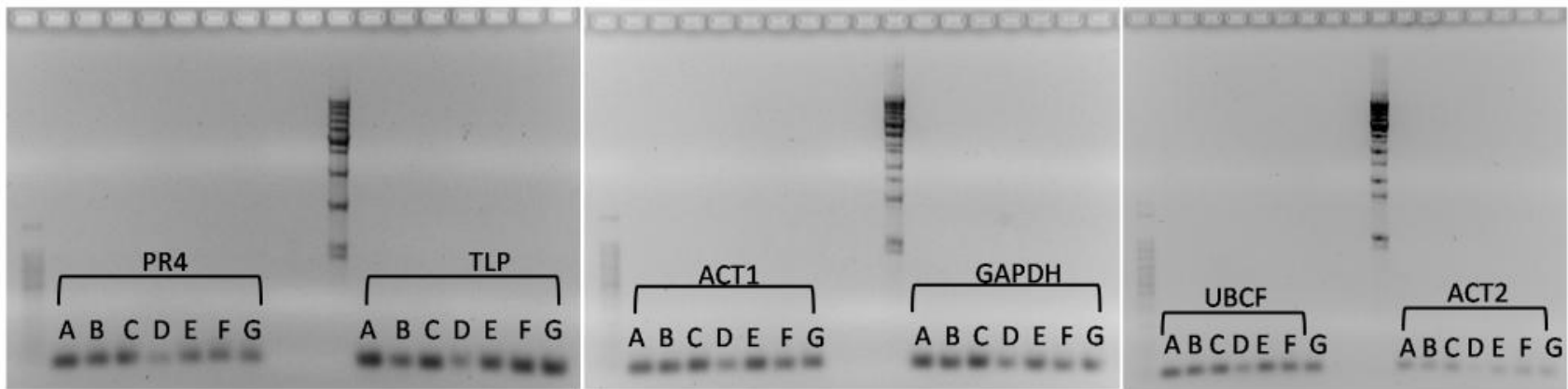


Figure A.2: RT-PCR products amplified from seven species of *Pinus* using primer pairs based on *P. taeda* genes. Template: cDNA prepared from RNA isolated from needles incubated in water (control conditions). Initial concentrations of template were not standardized, which accounts for variations in spot density. Amplification ran for 30 cycles of conditions described in (4). Lanes on far left and in the middle contain 100bp and 1kbp standards, respectively. Amplified genes are noted above the braces. Pine species were as follows: lane A, *P. sylvestris*; B, *P. strobus*; C, *P. pungens*; D, *P. virginiana*; E, *P. echinata*; F, *P. taeda* (positive control); G, *P. palustris*.

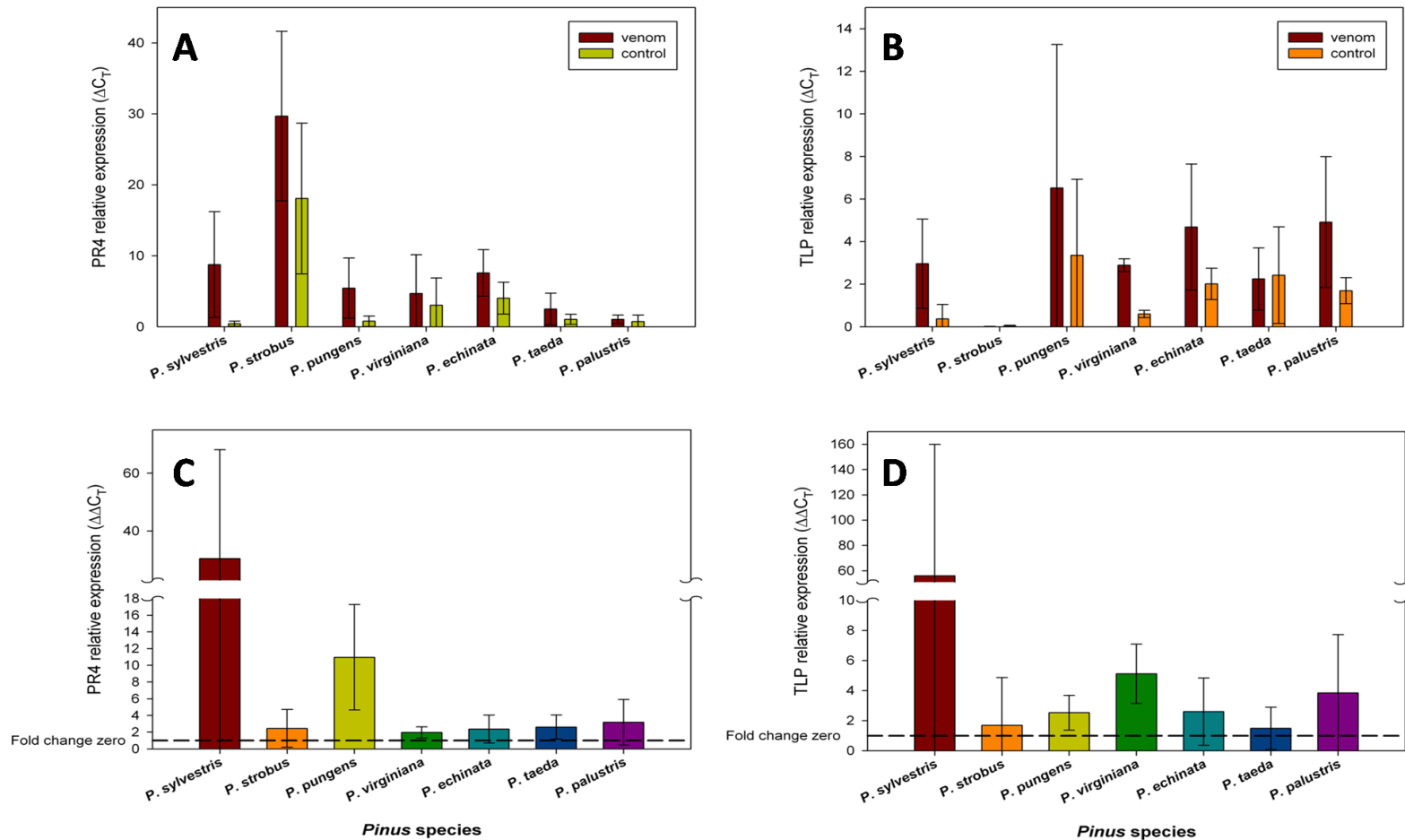


Figure A.3: Expression levels of biomarker genes in pine needles treated with *S. noctilio* venom. Needles from the same tree were treated either with an aqueous solution of *S. noctilio* venom or water, and gene expression levels were quantified using qRT-PCR. Panels A and B show relative expression for both venom-treated and water control needles. Panels C and D use fold change (ratio of treatment response to control response) to display the same data. In these figures the dashed line represents a ratio of one, signifying no change in relative expression. Panels A, C, PR4 expression; panels B, D, TLP expression. Note differences between y-axis scales in all panels.

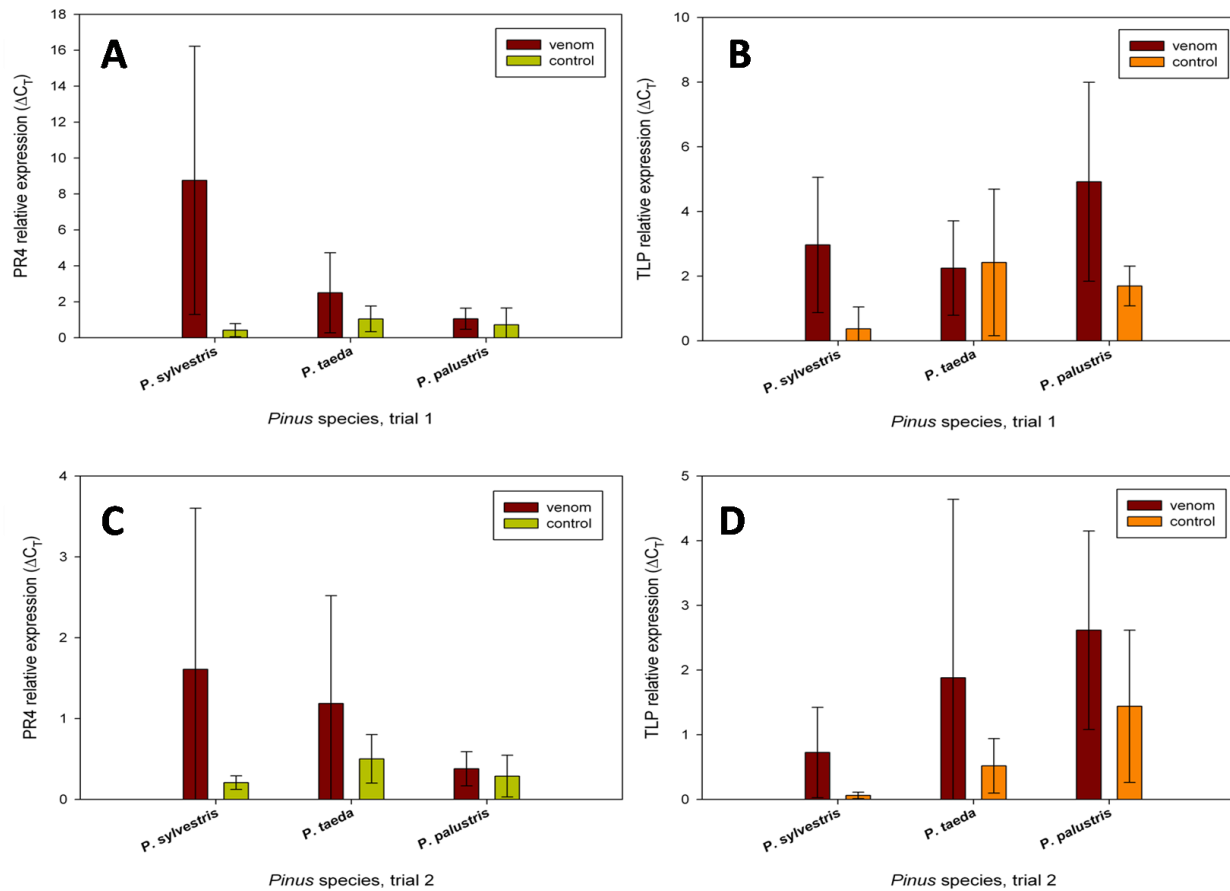


Figure A.4: Apparent biomarker gene expression responses to *S. noctilio* venom in needles collected on different dates via qRT-PCR. Results from the first collection date (February 2012) appear in panels A and B, while results from the second collection date (March 2012) appear in panels C and D. Data are shown only for the three species (Scots, loblolly, and longleaf pines) that yielded fully-replicated data at both time points, and the data shown in panels A and B are a subset of the data in Figs. A.3A, A.3B. Trees sampled were N=5 in Trial 1, and N=6 in Trial 2 for *P. taeda* and *P. palustris*, respectively, and N=3 for *P. sylvestris* for both trials. Panels A and C show PR4 expression relative to a water control, while panels B and D show relative TLP expression. Note differences between y-axis scales in all panels.

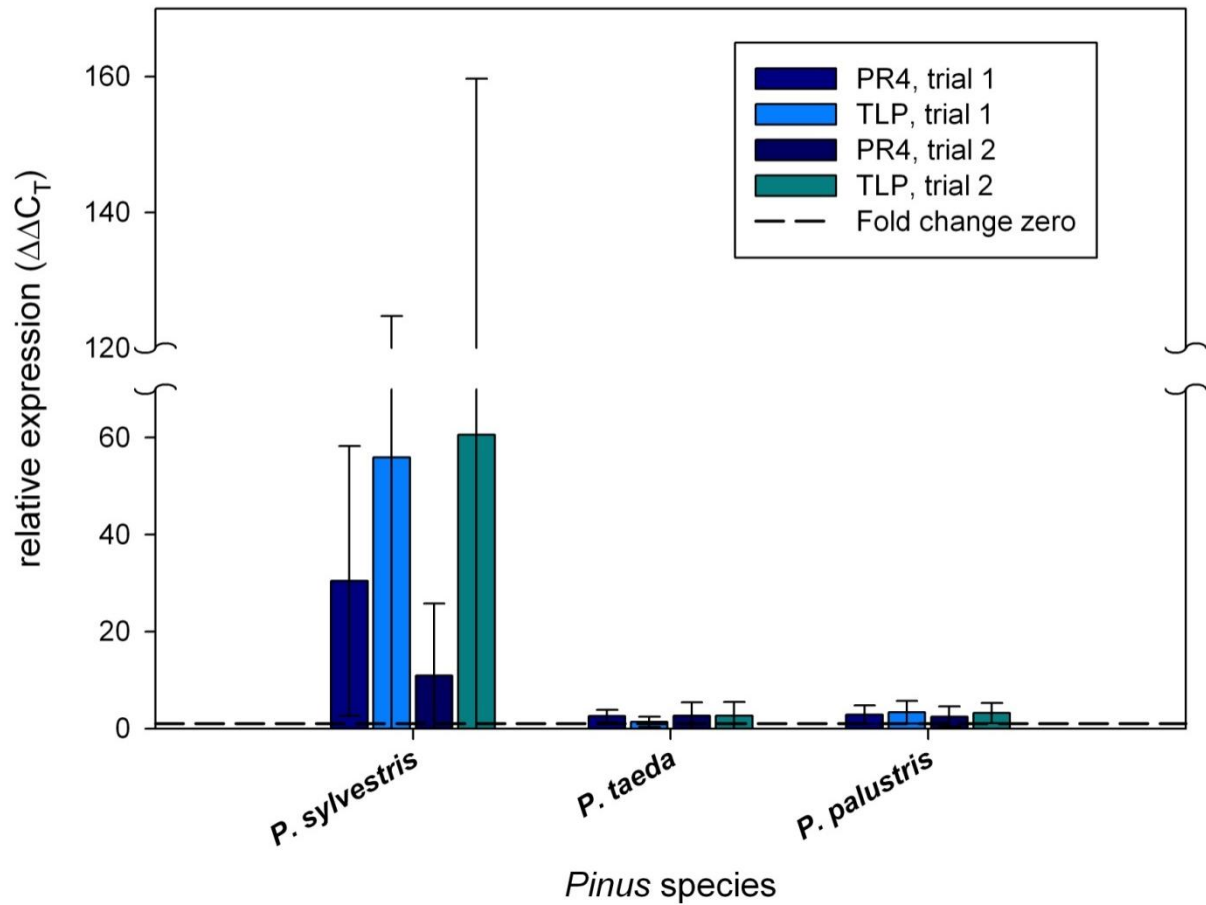


Figure A.5: Fold-change in expression of biomarker genes in three pine species responding to *S. noctilio* venom via qRT-PCR. The dashed line represents a ratio of one, signifying no fold-change difference in relative expression. Trees sampled were N=5 in Trial 1, and N=6 in Trial 2 for *P. taeda* and *P. palustris*, respectively, and N=3 for *P. sylvestris* for both trials.

References

1. Borchert, D., Fowler, G., and Jackson, L. (2006) Organism pest risk analysis: risk to the conterminous United States associated with the woodwasp, *Sirex noctilio* Fabricius, and the symbiotic fungus, *Amylostereum areolatum* (Fries: Fries) Boidin. USDA-APHIS-PPQ-EDP, Raleigh, NC USA
2. Dinkins, J. (2011) *Sirex noctilio* host choice and no-choice bioassays: woodwasp preferences for southeastern US pines. MS MS, University of Georgia
3. Fong, L. K., and Crowden, R. K. (1973) Physiological effects of mucus from wood wasp, *Sirex noctilio* F., on foliage of *Pinus radiata* D. Don. *Aust. J. Biol. Sci.* **26**, 365-378
4. Bordeaux, J. M., Lorenz, W. W., and Dean, J. F. D. (2012) Biomarker genes highlight intraspecific and interspecific variations in the responses of *Pinus taeda* L. and *Pinus radiata* D. Don to *Sirex noctilio* F. acid gland secretions. *Tree Physiol.* **32**, 1302-1312
5. Krutovsky, K. V., Elsik, C. G., Matvienko, M., Kozik, A., and Neale, D. B. (2007) Conserved ortholog sets in forest trees. *Tree Genet. Genomes* **3**, 61-70
6. Barbara, T., Palma-Silva, C., Paggi, G. M., Bered, F., Fay, M. F., and Lexer, C. (2007) Cross-species transfer of nuclear microsatellite markers: potential and limitations. *Mol. Ecol.* **16**, 3759-3767
7. Lesser, M. R., Parchman, T. L., and Buerkle, C. A. (2012) Cross-species transferability of SSR loci developed from transcriptome sequencing in lodgepole pine. *Mol. Ecol. Resour.* **12**, 448-455
8. Lorenz, W. W., Yu, Y.-S., and Dean, J. F. D. (2010) An improved method of RNA isolation from loblolly pine (*P. taeda* L.) and other conifer species. *J. Vis. Exp.*, e1751

9. Atkinson, N. J., and Urwin, P. E. (2012) The interaction of plant biotic and abiotic stresses: from genes to the field. *J. Exp. Bot.* **63**, 3523-3543
10. Richards, C. L., Rosas, U., Banta, J., Bhambhra, N., and Purugganan, M. D. (2012) Genome-wide patterns of *Arabidopsis* gene expression in nature. *PLoS Genet.* 10.1371/journal.pgen.1002662

APPENDIX B

PRELIMINARY PROTEOMIC ANALYSIS OF SIREX NOCTILIO VENOM AND CORRELATION WITH VENOM TRANSCRIPTOMIC DATA

B.1 Introduction

Insect venoms are complex mixtures of biologically-active compounds: enzymes, peptides, and small molecules (1-11). Due to their typical complexity, reliable analysis of these venoms typically involves application of multiple orthogonal approaches: analytical and preparative HPLC; biochemical fractionation and polyacrylamide gel analysis; bioassays, enzymology, and chemical identification; molecular cloning; and high-throughput genomic, transcriptomic, proteomic, and bioinformatic approaches (6,11-20). In recent years the combination of high-throughput sequencing (made increasingly inexpensive with next-generation techniques) coupled with the shotgun proteomics approach has yielded large, complex data sets. Transcriptomic data from messenger RNA offers a large picture of potential polypeptide components, but these data are made much more reliable and tractable when validated by the identification of actual translated polypeptides.

Several hymenopteran venoms have been interrogated using genomic, transcriptomic, and proteomic approaches in recent years (17,21,22), and some investigators have combined these approaches to resolve the venom (13,14,23). The field of comparative venomomics has benefited from the resulting nucleic acid and protein sequence data from several key species. To our knowledge, we present here the first comparison of transcriptomic and proteomic data for the

venom from a representative of the Symphyta, a phytophagous suborder of the Hymenoptera. We compare the results of a proteomic interrogation of the *Sirex noctilio* woodwasp venom tissue with the transcriptome of the tissue.

B.2 Materials and Methods

Venom sacs and glands (0.01 – 0.03 g each) were removed from four *S. noctilio* females and macerated separately in 0.5 ml millipure water to release protein, then lyophilized without heat. Deglycosylation was carried out using trifluoromethanesulfonic acid (TFMS), modifying a protocol by Edge et al. (24). Lyophilized protein was dissolved in 0.5 ml 2:1 TFMS:anisole (TFMS MP Biomedicals, Santa Ana, CA USA, anisole 99%, Sigma-Aldrich, St. Louis, MO USA) and the solution purged with nitrogen and shaken for 1 h. To this solution was added 0.5 ml cold anhydrous ether (Baker, Center Valley, PA USA) with mixing, followed by addition of 1 ml 50:50 pyridine:water (Sigma Aldrich, St. Louis, MO USA) with mixing. This solution was centrifuged to separate layers, and the ether layer was removed. The extraction was repeated, and the isolated aqueous layer was lyophilized and reconstituted in 0.5% TFA, 5% acetonitrile (TFA Acros, Morris Plains, NJ USA, acetonitrile, Fisher, Waltham MA USA). The protein solution was desalted using a Pierce C18 spin cartridge (Thermo, Waltham MA USA) following the manufacturer's instructions. The same procedure was carried out on a separate venom gland protein sample, substituting water for 2:1 TFMS:anisole as a native glycopeptide control. Purified, lyophilized protein was incubated with trypsin at 37°C overnight.

Mass spectrometry analyses were performed on a Thermo LTQ Orbitrap Elite mass spectrometer coupled with a Proxeon Easy NanoLC system (Waltham, MA) located at the Proteomics and Mass Spectrometry Facility, University of Georgia. Peptides were loaded onto a

5mm long and 300 μm id Dionex PepMap precolumn (Sunnyvale, CA), and then separated by a self-packed ~12 cm long, 100 μm id column/emitter with 200 \AA 5 μM Burker MagicAQ C18 (Auburn, CA), at 500 μL by gradient elution. Briefly, the two-buffer gradient (buffer A, 0.1% formic acid; buffer B, 99.9% acetonitrile, 0.1% formic acid) starts with 5% B, holds at 5% B for 2 min, then increases to 25% B over 60 min, to 40% B over 10 min, and to 95% B over 10 min. The Top 10 data-dependent acquisition (DDA) method was used to acquire MS data. A survey MS scan was acquired first, and then the top 10 ions in the MS scan were selected for CID. MS and MS/MS scans were then acquired at resolutions of 120,000 and 30,000, respectively. For the CID/ETD experiment, the Top 4 DDA method was used, alternating CID and ETD MS/MS. A total of four data files were generated, representing two methods of ionization each for both deglycosylated and native venom tissue.

The database of transcripts from *S. noctilio* woodwasp venom tissue generated previously (Chapter 4) was translated into FASTA files in all six reading frames, and the results from MS/MS scans above were used for BLASTp interrogation of this database (25). Hits in the database along with the peptide fragments identified were output from MASCOT software (Matrix Science, Boston, MA USA). These hits were then correlated to major findings from Chapter 4.

B.3 Results

The data generated from deglycosylated as well as native venom were complementary, as unique fragments were identified in many cases from one sample or the other. Additionally, CID-only data offered many hits within the database, and results from this ionization method were complemented by data from the CID/ETD ionization run to identify additional peptides

from the same sample. Pooled resulting peptide fragments gave high confidence in many of the resulting matches. These can be found in Table B.1. From the transcriptome database, only nine positive matches were found with high confidence. Among these were fragments corresponding to high-abundance unannotated transcripts. For the most abundant transcript, that corresponding to noctilisin, as well as the second most abundant transcript, no amino acid fragments were returned. Table B.1 lists the totals by reading frame and totals for the study with relative abundance values > 0.2 as given by MASCOT.

The top 20 proteins found in highest abundance within the venom proteome, ranked by correlated sequence transcript abundance, are listed in Table B.2. The most abundant protein identified is homologous to a fucosyl transferase in the bee *Apis florea*. A polypeptide N-acetylgalactosaminyltransferase most similar to one in the carpenter ant *Camponotus floridanus* is ranked fifth in abundance here. Other significant annotations include a copper transport protein homologous to one from the bee *Megachile rotundata* (ranked eighth), and an alcohol dehydrogenase from an ant, *Acromyrmex echinator*.

The third most abundant transcript from *S. noctilio* venom returned five unique fragments comprising a total of 115 amino acids in the first forward reading frame (Table B.3). For four of these fragments, the open reading frame positively identified by these fragments contained miscalled stop codons.

Five unique fragments were also identified in the first forward reading frame of the fifth most abundant venom transcript, comprising 122 unique amino acids. Two of the fragments contain an identical ten-amino acid stretch identical save for 1 amino acid, identified by nucleic acid sequencing as a stop codon. Four of the five fragments positively identify an amino acid miscalled as a stop codon by Illumina sequencing.

A single contiguous peptide comprised of 55 amino acids lying in the second forward reading frame was identified as corresponding to the sixth most abundant transcript in venom tissue.

Twenty-nine unique amino acid fragments comprising 457 unique amino acids in the second forward reading frame corresponded to the eighth abundance transcript, encoding an apparent laccase-like multicopper oxidase (LMCO). Two of the 29 peptide fragments were found upstream of the ORF containing the remaining 27 fragments (Table B.4).

Peptide fragments were identified corresponding to a highly-abundant transcript encoding an apparent elongation factor 1-alpha with high homology to one in *Nasonia vitripennis*. This transcript was the third in abundance among annotated transcripts. Four unique fragments comprising a total of 42 unique amino acids were identified, all within a single ORF in the first forward reading frame of the nucleotide sequence (Table B.5).

Peptide fragments were identified for the fourth most abundant annotated transcript encoding an apparent *Solenopsis invicta* (ant) protein. Seven fragments comprising 72 unique amino acids were identified. Two of the fragments were identical except for a single amino acid, both miscalled in the nucleic acid sequence as a stop codon. These two fragments plus one further (total of three fragments) were identified having amino acids that were apparently miscalled as stop codons by Illumina sequencing. All seven fragments were found in the first forward reading frame.

Eighteen amino acid fragments comprised of 260 unique amino acids corresponded to a relatively low-abundance sequence (140 TPM) encoding an apparent amylase. Earlier literature reported evidence of amylase activity, and this amylase was identified within the transcriptome in Chapter 4. Each of these fragments was found to lie within a single ORF in the first forward

reading frame identified as corresponding through sequence homology to an amylase from *Bombus impatiens*.

Twelve fragments comprising 196 unique amino acids corresponded to a sequence for the second most abundant apparent protease transcript, with homology to a carboxypeptidase D-like protein in *Megachile rotundata*. All were found in the first forward reading frame, and two of the twelve fell outside the defined ORF containing all others, one upstream and one downstream, indicating that the nucleic acid sequencing may contain some miscalled stop codons.

B.4 Discussion

Several useful insights into the nature of the transcriptomic dataset have been made available to us through the use of shotgun proteomics. Among these, identification of the correct single reading frame by amino acids may be the most important. Future studies requiring heterologous expression of protein products will rely on this information.

Secondly, proteomic data indicated that fragments corresponding to two of the most abundant transcripts (ranked third and fifth) contain miscalled stop codons. Based on this information, the longest open reading frames identified in unannotated transcripts in Chapter 4 may be unreliable.

The highly abundant fucosyltransferase, both as a transcript and as a protein, is interesting. Given what we know about glycan presence in venom, and its characterization as a mucopolysaccharide-protein complex in early literature, it is reasonable to find an enzyme with such activity within the venom tissue.

Significantly, one of the most abundant proteins present in venom is necessary to modify another important venom component, noctilisin, with its known substituent, N-

acetylglucosamine. Given the importance of this glycopeptide for the activity of venom on pines, and the lack of activity when noctilisin is not glycosylated, this is not a surprising finding. As venom was not isolated from venom glandular tissue, it is impossible to ascertain whether this enzyme exerts its activity in the venom or merely in the venom glandular tissue.

The most abundant transcript identified in the venom, that containing the glycopeptide noctilisin, returned no matching protein fragments. Given its abundance (nearly half of all reads from *S. noctilio* venom were associated with this transcript), it would seem at first glance surprising that no protein fragments were detected by proteomic analysis. In fact, this result was anticipated: during the protein clean-up step prior to MS analysis, the final C18 spin column wash to remove acid and salt was performed using five percent acetonitrile in water. Noctilisin, a small, highly water-soluble small glycopeptide, eluted from a C18 column at a concentration of acetonitrile less than five percent. Thus it was not surprising that these fragments could not be identified in the protein, as they were washed away during sample cleanup. Further, we did not demonstrate the effectiveness of deglycosylation procedures on the isolated peptide. We anticipate that the second most abundant transcript also codes for a highly water-soluble peptide. Further study using alternative clean-up procedures prior to analysis are likely to reveal abundant presence of noctilisin. As noctilisin has already been fully characterized structurally, this analysis was made more effective and informative by its exclusion from the analysis. Less abundant species were likely to be missed in the presence of a highly-abundant species.

Interestingly, three species with high numbers of individual fragments as well as relatively high TPM values all were found from enzymes: the LMCO above, an amylase, and an apparent protease. Each of these activities was identified in earlier literature.

In Chapter 4, laccase activity was demonstrated from venom via zymogram. In this case, the high abundance of the transcript corresponded to an abundant protein. Validation for the abundant presence of a phenoloxidase (the LMCO of eighth abundance among transcripts) was gratifying given its tentative identification in early literature and positive identification of activity in Chapter 4. Within the venom transcriptome, this sequence was well-represented among the findings from proteomic analysis. The two fragments found upstream of the ORF containing the other 27 fragments could represent another example of sequencing miscall as a stop codon. The investment of *S. noctilio* in this expensive enzyme indicates its importance for the wasp, the offspring, or its fungal symbiont, though the functional significance of the enzyme remains poorly understood.

Another important insight we take from this analysis is the lack of one-to-one correspondence between abundance of transcripts and abundance of protein. It is clearly not the case for the amylase, with an abundance of 140 TPM for its transcript, versus 6,663 TPM for the LMCO above, yet both are apparently abundantly translated.

Several miscalls for stop codons were identified in the protease yielding peptide fragments. Our data confirmed that a combined approach, validating transcriptomic sequence by proteomic analysis, is an effective approach for ensuring reliable results. The transcriptomic sequence data has been shown to be highly reliable and useful given the number of fragments mapping directly onto nucleic acid sequence data.

Table B.1: Transcriptome sequences with corresponding proteomic hits having relative expression values > 0.2. Each reading frame combines all forward and reverse hits from both deglycosylated and native venom protein, also combining CID only data with CID/ETD/HSD.

Combined reading frame 1	89
Combined reading frame 2	84
Combined reading frame 3	264
Total sequences identified	437

Table B.2: Protein abundance correlated with transcript abundance. Transcript relative abundance expressed as number of transcripts per million (TPM) among all transcripts, including those with ribosomal annotations. Protein relative expression is ranked per reading frame, both forward and backward.

Sequence Id	TPM	Venom transcriptome abundance ranking	Proteome relative expression ranking	Relative expression value	Frame	Annotation
comp9077_c0_seq1	889.9	38	20	10	2	alpha-(1,3)-fucosyltransferase C-like [<i>A. florea</i>]
comp7084_c0_seq1	157.1	144	19	10	1	alpha,alpha-trehalose glucohydrolase (trehalase) [<i>P. hypochondriaca</i>]
comp5140_c0_seq1	154.9	147	105	10	3	60S ribosomal protein L35-like [<i>A. florea</i>]
comp12853_c0_seq1	73.3	226	12	10	3	replication-associated protein A, partial [<i>E. coli</i> PA28]
comp10716_c6_seq1	66.0	236	22	10	3	putative polypeptide N-acetylgalactosaminyltransferase [<i>B. impatiens</i>]
comp5114_c0_seq1	53.2	276	7	10	2	phosphoserine aminotransferase [<i>C. floridanus</i>]
comp7210_c0_seq1	38.7	349	36	10	3	slit homolog 3 protein-like [<i>B. terrestris</i>]
comp7212_c0_seq1	36.6	363	50	10	3	copper transport protein ATOX1-like [<i>M. rotundata</i>]
comp9119_c0_seq2	34.6	382	90	10	3	EH domain-containing protein 1-like [<i>A. florea</i>]
comp3670_c0_seq1	25.3	514	10	10	2	Alcohol dehydrogenase [NADP+] A [<i>A. echinator</i>]
comp3525_c0_seq1	17.8	680	3	10	1	moesin/ezrin/radixin homolog 1-like [<i>M. rotundata</i>]
comp8630_c1_seq5	17.8	681	6	10	1	short/branched chain specific acyl-CoA dehydrogenase, mitochondrial [<i>C. floridanus</i>]
comp10362_c0_seq1	17.5	690	51	0.7925	1	GAPDH [NAD+], cytoplasmic [<i>H. saltator</i>]
comp3791_c0_seq1	17.3	705	19	10	2	proclotting enzyme [<i>A. echinator</i>]
comp17588_c0_seq1	17.0	712	165	10	3	small glutamine-rich tetratricopeptide repeat-containing protein alpha-like [<i>B. terrestris</i>]
comp3495_c0_seq1	15.6	763	4	10	2	peroxisomal N(1)-acetyl-spermine/spermidine oxidase-like [<i>A. florea</i>]
comp17171_c0_seq1	14.3	830	23	10	2	small ubiquitin-related modifier-like [<i>B. terrestris</i>]
comp3226_c0_seq1	11.8	988	99	10	3	heat shock factor-binding protein 1-like [<i>B. impatiens</i>]
comp6783_c0_seq1	11.4	1021	20	10	1	something about silencing protein 10-like [<i>M. rotundata</i>]
comp3604_c0_seq1	10.7	1082	29	10	3	KLRAQ motif-containing protein 1-like [<i>B. terrestris</i>]

Table B.3: Proteomic data correlated with significant transcript abundance. Data ranked by transcript abundance correlated with relatively high abundance in the proteomic analysis. Transcript relative abundance expressed as number of transcripts per million (TPM) among all transcripts, including those with ribosomal annotations. Sequences with TPM values < 10 were excluded. Protein relative expression is ranked per reading frame, both forward and backward.

Sequence Id	TPM	Venom transcriptome abundance ranking	Proteome relative expression ranking	Relative expression value	Frame	Annotation
comp3255_c1_seq1	112735	3	41	0.93	1	--
comp5157_c0_seq1	35349	5	73	1.36	1	--
comp11331_c0_seq1	13057	6	75	0.41	2	--
comp9052_c0_seq1	6663	8	50	0.23	2	laccase-like multicopper oxidase [<i>A. gambiae</i>]
comp7127_c0_seq1	1947	13	27	1.29	1	elongation factor 1-alpha [<i>N. vitripennis</i>]
comp11322_c1_seq1	1799	15	50	0.79	1	hypothetical protein SINV_04326 [<i>S. invicta</i>]
comp10913_c0_seq1	140	160	82	0.31	1	alpha-amylase-like [<i>B. impatiens</i>]
comp15972_c0_seq1	26	490	86	0.23	1	carboxypeptidase D-like [<i>M. rotundata</i>]

Table B.4: Peptide fragments obtained for an apparent LMCO in *S. noctilio* woodwasp venom.

Sequence Id	Significance	Frame	TPM
comp9052_c0_seq1	8th most abundant transcript LMCO [<i>A. gambiae</i>]	+2	6663
Fragments	Found in BLAST ORF?		
RSVVEIIFRDLSSNNPAVAHPFHLHGYKF	yes		
RYVFPVYDVGTYFYHSHAGFHRDEGIFGALIVRQ	yes		
RQNPDLNVGLLKN	yes		
KSFDPIDNEERD	yes		
KYYSFHQLDRRT	yes		
KGHVPALPPNFPKC	yes		
KMTNVPSANGIPALRD	yes		
RSPGIFPYSFLINGYGRY	yes		
RDLSNNPAVAHPFHLHGYKF	yes		
RYVFPVYDVGTYFYHSHAGFHRD	yes		
RTSNETTPTPLATFHVKT	yes		
KTGSSYRF	yes		
RDTVMIKRG	yes		
RVDFVLNATKT	yes		
RVKGHVPALPPNFPKC	yes		
KFYVMEMVQGNETLKM	yes		
KNVEDEREHVMVLSDYMHDTATMFFPGLPSRS	yes		
RGGATRV	yes		
KTGVTAKS	yes		
RSVVEIIFRD	yes		
KSINRRH	yes		
RQNPDLNVGLLKN	yes		
KTGVTAKSIVNSRN	yes		
KMTNVPSANGIPALRD	yes		
RTSNETTPTPLATFHVKT	yes		
RSYKMSVXPAXKH	two Xs are stops forward of defined ORF		
RHVGPDIIQICYGDLVVVKV	yes		
RTLHSGAINNISIFPPEVSMNRRN	yes		
RAIFFRX	first A, last X are stops forward of defined ORF		

Table B.5: Peptide fragments obtained for an alpha amylase homolog in *S. noctilio* woodwasp venom.

Sequence Id	Significance	Frame	TPM
comp10913_c0_seq1	1st abundant amylase: alpha-amylase-like [<i>B. impatiens</i>]	+1	140

Fragments	Found in BLAST ORF?
RIMSSYRF	yes
RSGNEIAFRD	yes
RYQPISYKW	yes
KDLTAIYGRL	yes
KMDYVNIAAVTEFKY	yes
RDAHDTMVHLFEWKF	yes
KMAIAFMLAHPYGTARI	yes
RGSVGFIAINADSWDLRQ	yes
KYGYEISNALHGNNLLKW	yes
KWVFVNWGEAWALVPSDALVFIDNHDNQRG	yes
RIDAAKH	yes
RDMVKRC	yes
KHMWPKD	yes
RIMSSYRF	yes
KIVNFMNAAIEAGVAGFRI	yes
RQRLQTCLPAGKY	yes
KYGYEISNALHGNNLLKW	yes
RFFGPIGYGGIQVSIQENVISSRPWWERY	yes

References

1. Casewell, N. R., Wüster, W., Vonk, F. J., Harrison, R. A., and Fry, B. G. (2013) Complex cocktails: the evolutionary novelty of venoms. *Trends in Ecol. Evol.* **28**, 219-229
2. de Lima, P. R., and Brochetto-Braga, M. R. (2003) Hymenoptera venom review focusing on *Apis mellifera*. *J. Venom. Anim. Toxins* **9**, 149-162
3. Hider, R. C. (1988) Honeybee venom: a rich source of pharmacologically active peptides. *Endeavour* **12**, 60-65
4. Hoffman, D. R. (2010) Ant venoms. *Curr. Opin. Allergy Clin. Immunol.* **10**, 342-346
5. Lai, R., and Liu, C. (2011) Bioactive peptides and proteins from wasp venoms. in *Toxins and Hemostasis* (Kini, R. M., Clemetson, K. J., Markland, F. S., McLane, M. A., and Morita, T. eds.), Springer, Dordrecht. pp 83-95
6. Leluk, J., Schmidt, J., and Jones, D. (1989) Comparative studies on the protein composition of hymenopteran venom reservoirs. *Toxicon* **27**, 105-114
7. Palma, M. S. (2006) Insect venom peptides. in *Handbook of Biologically Active Peptides* (Kastin, A. J. ed.), Academic Press, New York. pp 389-396
8. Piek, T. (1990) Neurotoxic kinins from wasp and ant venoms. *Toxicon* **29**, 139-149
9. Piek, T., and Spanjer, W. (1986) *Chemistry and pharmacology of solitary wasp venoms*,
10. Schmidt, J. O. (1982) Biochemistry of insect venoms. *Annu. Rev. Entomol.* **27**, 339-368
11. Zalat, S. M. (1997) Vespidae venom analysis with phylogenetic inferences. *Biochem. Syst. Ecol.* **25**, 767-774
12. Asgari, S., and Rivers, D. B. (2011) Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. in *Annual Review of Entomology, Vol 56* (Berenbaum, M. R., Carde, R. T., and Robinson, G. E. eds.), Annual Reviews, Palo Alto. pp 313-335

13. de Graaf, D. C., Aerts, M., Brunain, M., Desjardins, C. A., Jacobs, F. J., Werren, J. H., and Devreese, B. (2010) Insights into the venom composition of the ectoparasitoid wasp *Nasonia vitripennis* from bioinformatic and proteomic studies. *Insect Mol. Biol.* **19**, 11-26
14. de Graaf, D. C., Aerts, M., Danneels, E., and Devreese, B. (2009) Bee, wasp and ant venomomics pave the way for a component-resolved diagnosis of sting allergy. *J. Proteomics* **72**, 145-154
15. Magalhaes de Abreu, R. M., Morelli Silva de Moraes, R. L., and Camargo-Mathias, M. I. (2010) Biochemical and cytochemical studies of the enzymatic activity of the venom glands of workers of honey bee *Apis mellifera* L. (Hymenoptera, Apidae). *Micron* **41**, 172-175
16. Piek, T. (ed) (1986) *Venoms of the Hymenoptera: biochemical, pharmacological and behavioural aspects.*, Academic Press, London
17. Pinto, J., Fox, E. G. P., Saidemberg, D. M., Santos, L. D., Menegasso, A. R. D., Costa-Manso, E., Machado, E. A., Bueno, O. C., and Palma, M. S. (2012) Proteomic view of the venom from the fire ant *Solenopsis invicta* Buren. *J. Proteome Res.* **11**, 4643-4653
18. Schachter, M., and Thain, E. M. (1954) Chemical and pharmacological properties of the potent, slow contracting substance (kinin) in wasp venom. *British Journal of Pharmacology and Chemotherapy* **9**, 352-359
19. Schmidt, J. O., Blum, M. S., and Overall, W. L. (1986) Comparative enzymology of venoms from stinging Hymenoptera. *Toxicon* **24**, 907-921

20. Wong, L. K., and Crowden, R. K. (1976) Preliminary studies on mucus secretion of wood wasp, *Sirex noctilio* F .1. Physicochemical and biochemical properties. *Aust. J. Biol. Sci.* **29**, 21-32
21. Baracchi, D., and Turillazzi, S. (2010) Differences in venom and cuticular peptides in individuals of *Apis mellifera* (Hymenoptera: Apidae) determined by MALDI-TOF MS. *J. Insect Physiol.* **56**, 366-375
22. Bouzid, W., Klopp, C., Verdenaud, M., Ducancel, F., and Vétillard, A. (2013) Profiling the venom gland transcriptome of *Tetramorium bicarinatum* (Hymenoptera: Formicidae): the first transcriptome analysis of an ant species. *Toxicon* **70**, 70-81
23. Vincent, B., Kaeslin, M., Roth, T., Heller, M., Poulain, J., Cousserans, F., Schaller, J., Poirie, M., Lanzrein, B., Drezen, J.-M., and Moreau, S. (2010) The venom composition of the parasitic wasp *Chelonus inanitus* resolved by combined expressed sequence tags analysis and proteomic approach. *BMC Genomics* **11**, 693
24. Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E., and Weber, P. (1981) Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal. Biochem.* **118**, 131-137
25. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410