Noctilisin, a Venom Glycopeptide of *Sirex noctilio* (Hymenoptera: Siricidae), Causes Needle Wilt and Defense Gene Responses in Pines

J. MICHAEL BORDEAUX,[1] W. WALTER LORENZ,[1] DARRYL JOHNSON,[2] MAJORS J. BADGETT,[2] JOHN GLUSHKA,[3] RONALD ORLANDO,[3,4] AND JEFFREY F. D. DEAN[1,4,5]

J. Econ. Entomol. 107(5): 1931–1945 (2014); DOI: http://dx.doi.org/10.1603/EC14151

ABSTRACT During oviposition, female *Sirex noctilio* (F.) (Siricidae) woodwasps inject their conifer hosts with a venom gland secretion. The secretion induces a variety of host 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 reversed-phase–high-performance liquid chromatography using activity assays based on defense gene induction as well as the needle wilt response in pine shoot explants. An 11-amino acid, posttranslationally modified peptide (SEGPROGTKRP) encoded by the most abundant transcript recovered from *S. noctilio* venom gland tissue comprised the backbone of the 1,850 Da active factor. Posttranslational 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 contains motifs which may explain how it avoids detection by pine defense systems.

KEY WORDS drocosin, glycopeptide, phosphoethanolamine-modified GalNAc, Tn antigen, venom

Sirex noctilio (F.) (Siricidae), 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 (Slippers et al. 2012). *S. noctilio* has recently become established in the region around Lake Ontario (Dodds et al. 2010, Dodds and de Groot 2012), 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 used by plants to recognize and react to insect pests.

S. noctilio attacks conifers as part of the reproductive process for these insects (Talbot 1977). 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* (Chaillet ex Fr.) Boidin. 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 (Fong and Crowden 1973). 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 (Coutts 1969a). With the tree in a state of weakened defense from envenomation, the oidia spores of *A. areolatum* initiate mycelial growth and the fungus spreads, colonizing the surrounding wood. Af-

[1] From the Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA 30602.
[2] The Department of Chemistry, University of Georgia, Athens, GA 30602.
[3] The Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602.
[4] The Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602.
[5] Corresponding author, e-mail: jeffdean@uga.edu.

0022-0493/14/1931–1945$04.00/0

ter the eggs hatch, *S. noctilio* larvae tunnel through the wood, deriving nutrition from fungus-altered wood. After 1–2 yr 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 (Madden 1968, 1975).

Early studies of the *S. noctilio* interaction with pines identified several effects and responses that were associated with specific components of the pathosystem (Coutts 1969a,b). 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 (Wong and Crowden 1976, Bordeaux and Dean 2012). 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 (Coutts 1969a).

In general, hymenopteran (apocritan) venoms are highly variable mixtures of bioactive polypeptides and small molecules (Piek 1986, Danneels et al. 2010, Asgari and Rivers 2011, Colinet et al. 2013, Moreau 2013); 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 (Boros 1968, Wong and Crowden 1976). 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 (Coutts 1970, Wong and Crowden 1976). However, sensitivity to protease digestion (Bordeaux et al. 2012) suggested that the active factor might be a bioactive peptide, particularly as many such molecules have been found in other hymenopteran venoms (Vetter et al. 2011). Quantitative real-time–polymerase chain reaction (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 (Bordeaux et al. 2012) were used to follow the wilt-inducing factor during venom fractionation. Purification and characterization of the active factor are described here.

Materials and Methods

Purification and Activity Testing. Venom glands were extracted from *S. noctilio* females provided by Kelley Zylstra (U.S. Department of Agriculture–Animal and Plant Health Inspection Service [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 before storage at −20°C. After thawing, the solution was centrifuged at 17,000 × *g* for 5–10 min, and the clarified supernatant was retained (Fraction 2). Using a centrifugal concentrator (Pall, Ann Arbor, MI), the supernatant was concentrated against a 10-kDa molecular weight cutoff (MWCO) membrane at 6,700 × *g*, and the pass-through volume was retained (Fraction 3). Reversed-phase–high-performance liquid chromatography (RP-HPLC) separation of Fraction 3 was carried out using a Zorbax Eclipse Plus C18 column, 4.6 by 250 mm, 5 μm (Agilent, Santa Clara, CA). Chromatographic conditions were as follows: the flow rate was 1.2 ml/min, mobile phase A was 0.07% trifluoroacetic acid (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-μl injection was lyophilized (Eppendorf Vacufuge, Hamburg, Germany) and resuspended in 250 μl of reagent-grade water. The activity contained in each fraction was measured by dosing individual shoot tips from sensitive *Pinus radiata* D. Don plants maintained in a growth chamber with a 50-μl aliquot of the water suspension followed by 24-h incubation. Procedures for RNA extraction, cDNA synthesis, and qRT-PCR quantitation were described previously (Bordeaux et al. 2012). Briefly, 5 μl of 0.5 μM primer pair, 5 μl of 0.5 μM cDNA template, and 10 μl of SybrGreen 2× Supermix (Bio-Rad, Hercules, CA) were mixed, and the 20 μl reactions were run on an iCycler thermal cycler (Bio-Rad, 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, and 55°C for 1 min. Postamplification melt-curve analysis used 81 cycles from 55° to 95°C for 10 s, incremented at 0.5°C. All qRT-PCR procedures used 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% relative standard deviation (RSD).

At various stages of the purification process, results from the qRT-PCR assays were validated against a needle-wilt bioassay. Briefly, 3-mo-old *P. radiata* seedlings were cut at the soil line and dosed with 50 μl of purified factor (97 μM noctilisin), 20 mg/ml of 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.

Molecular Weight Determination. The active factor recovered from RP-HPLC was subjected to mass spectrometry analysis using an AB SCIEX TOF/TOF 5800 System (AB SCIEX, Framingham, MA) by suspending 7.5 mM peptide (50× whole venom

equivalent) in a matrix consisting of α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) and a vol:vol 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 ($CGRP_{8-37}$, MW 3125.59 Da). A separate aliquot of 7.5 mM concentrated peptide was subjected to C18 capillary separation (Halo Peptide ES-C18, 150 × 200 μm, 5 μm Advanced Materials Technology, Wilmington, DE) with electrospray ionization mass spectrometry (Finnegan LTQ, Thermo Fisher, Waltham, MA). The $M + 2H^+$ fragment m/z 925 (peak corresponding to 1,850 Da) was chosen for fragmentation via collision-induced dissociation.

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 (Wellner et al. 1990).

The *S. noctilio* venom gland transcriptome characterized using Illumina DNA sequencing to generate >24,000 unique transcript assemblies was previously described (J.M.B., unpublished data). 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 (Rozen and Skaletsky 2000). 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 amplimer product was sequenced in the Georgia Genomics Facility at University of Georgia (UGA).

Peptide Synthesis. As total synthesis of mature noctilisin was impractical, a synthetic peptide backbone (nonglycosylated SEGPROGTKRP, 1,197 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 nonglycosylated synthetic peptide (aglyconoctilisin) versus the glycopeptide purified from venom glands. In these assays, 50 μl aliquots of 30 μM and 300 μM of synthetic peptide solutions in water were applied to excised shoot tips.

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). Assuming a per-residue molar coefficient of 10,000/M × cm for amino acid residues, the 11-amino acid synthetic peptide was calculated to have $\epsilon_{190} = 110{,}000$ (Hennessey and Johnson 1981, Kingston et al. 2005). 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. Aliquots (50 μl) 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 to 97 μM (0.33–4.9 nmoles applied to each shoot tip).

Nuclear Magnetic Resonance (NMR) Structural Characterization. Purified peptide (≈90 nmoles) was dissolved in 80 μl of D_2O to exchange all labile protons. Standard 2D correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), rotating frame nuclear Overhauser effect spectroscopy (ROESY), and heteronuclear single quantum coherence (HSQC) spectra were collected on an Agilent 600 MHz DD2 spectrometer (Agilent, Santa Clara, CA) equipped with a 3-mm cryogenic probe at 25°C. A 1D $(^1H)^{31}P$ heteronuclear multiple quantum coherence (HMQC) experiment was collected using a Varian Inova 500 MHz spectrometer (Agilent, Santa Clara, CA) with an HX probe tuned to ^{31}P. The sample was then exchanged with H_2O and a 500 ms mixing time nuclear Overhauser effect spectroscopy (NOESY) dataset was collected using a 900 MHz DD2 spectrometer. Data were processed using Mnova software (Mestrelab Inc. Escondido, CA).

Results

Purification and Molecular Weight Determination. RP-HPLC separated the heat-treated venom gland Fraction 3 (10-kDa MWCO pass-through in the "Purification and Activity" section above) into numerous peaks of various sizes detected at 254 nm; activity was limited to the third and fourth fractions between 40 and 60 min (Fig. 1). Monitoring at 280 nm revealed nearly all the activity was confined to a single poorly absorbing peak at 60 min (Fig. 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 ≈60 min, corresponding to the low-absorbing peak at higher wavelengths (Fig. 3).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) detected a single component (noctilisin; Fig. 4B) having an internal standard calibrated mass of 1849.95 Da (Fig. 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 collision-induced dissociation to yield doubly-charged fragments m/z 904.23, 864.27, and 762.71. These fragments corresponded with the loss of $-CH_2CH_2NH_2$, $-PO_3CH_2CH_2NH_2$, and -GalNac-$PO_3CH_2CH_2NH_2$ from the side chains, as will be discussed below in conjunction with NMR analyses.

Sequence Analysis. Edman degradation of the active peak component yielded amino acid sequence of

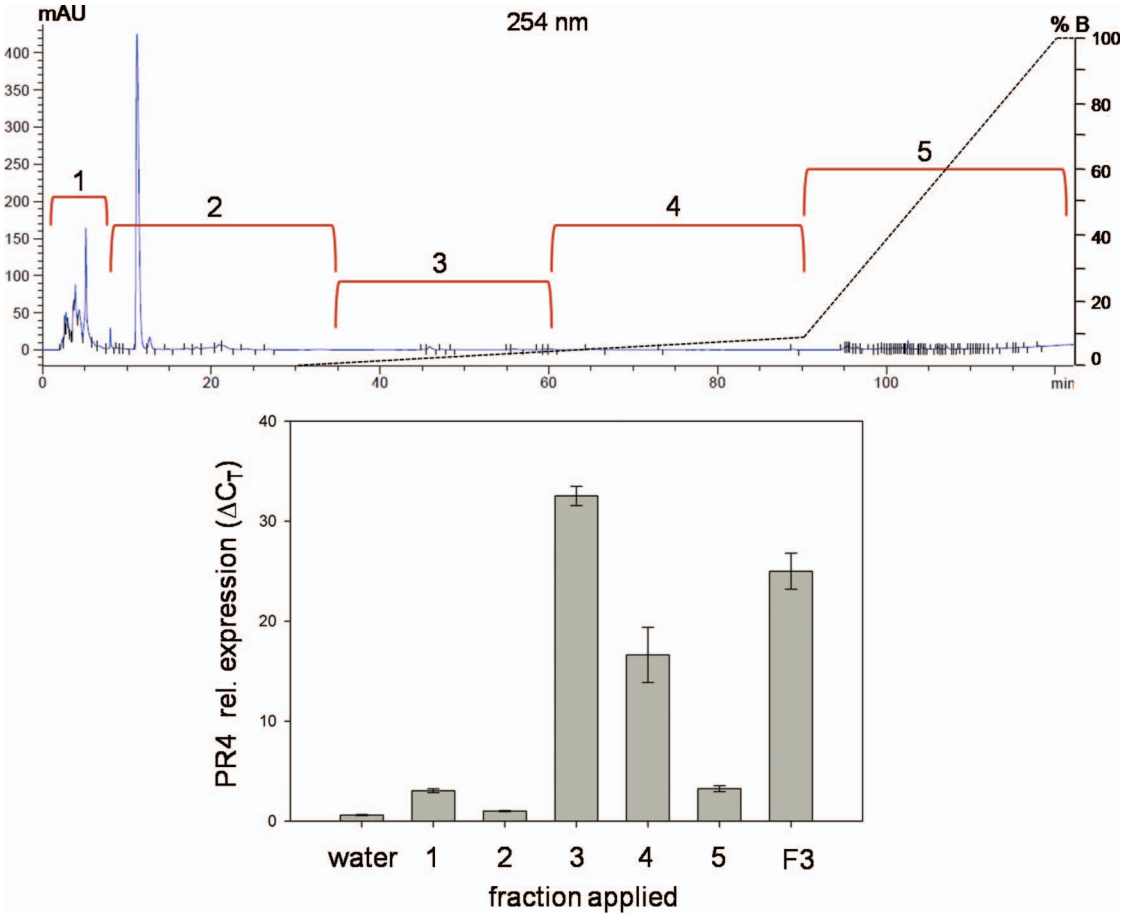


Fig. 1. RP-HPLC fractionation of venom 10-kDa pass-through and bioassay evaluation. Compounds in a 500-μl aliquot of *S. noctilio* venom Fraction 3 (F3) were separated using C18 RP-HPLC and following eluent absorbance at 254 nm. Eluent B was 0.04% TFA in acetonitrile. Noctilisin activity was essentially limited to the third and fourth fractions collected ($n = 3$). (Online figure in color.)

XEGPROGTKRP. Subsequent pretreatment of the peptide with acid and heat (Wellner et al. 1990) deblocked 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 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 bp in length and had a stop codon immediately after the terminal proline residue of the sequenced peptide (Fig. 5A) and a 23-amino acid signal peptide (Fig. 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 amplimer corroborated the nucleotide sequence predicted from the Illumina assemblies.

NMR Analysis. Purified noctilisin was subjected to a series of NMR analyses to further refine its structure. A 1D proton spectrum (Fig. 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 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 H_2O revealed seven amide protons with NOEs to α-protons of

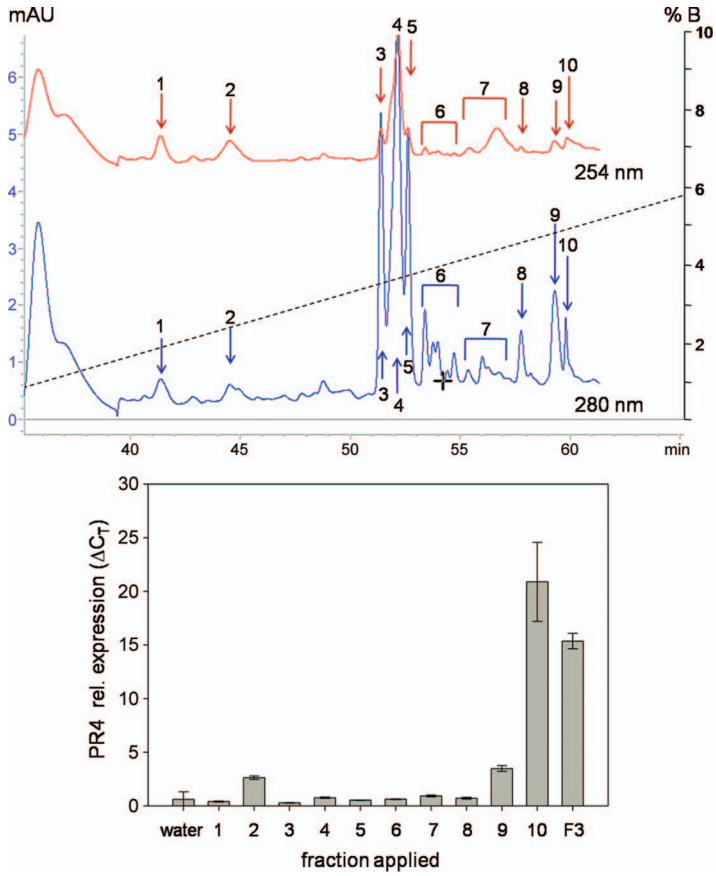


Fig. 2. RP-HPLC fractionation of venom 10 kDa pass-through to single components and bioassay evaluation. Compounds in a 500-μl aliquot of *S. noctilio* venom Fraction 3 (F3) were separated using C18 RP-HPLC and following eluent absorbance at both 254 and 280 nm. Eluent B was 0.04% TFA in acetonitrile. Activity was limited essentially to a single peak eluting at 59.6 min ($n = 3$). (Online figure in color.)

the previous residue, and so confirmed the sequence of fragments, $S_1E_2G_3$, P_4R_5, and $hP_6G_7T_8K_9R_{10}$. In addition, NOE crosspeaks were observed between the $G_3\alpha$ and $P_4\delta$ 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 Hβ protons in the ROESY spectrum (Fig. 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 P^{31}/H^1 correlation experiment (Fig. 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 with 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 because nearly all identifiable crosspeaks pointed to a most likely extended structure (Fig. 7), significant secondary structural features for noctilisin did not appear to be supported.

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 with the theoretical absorptivity predicted for an 11-amino acid peptide at this signature wavelength for peptide bonds. The two were in close agreement, and as 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 (Fig. 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

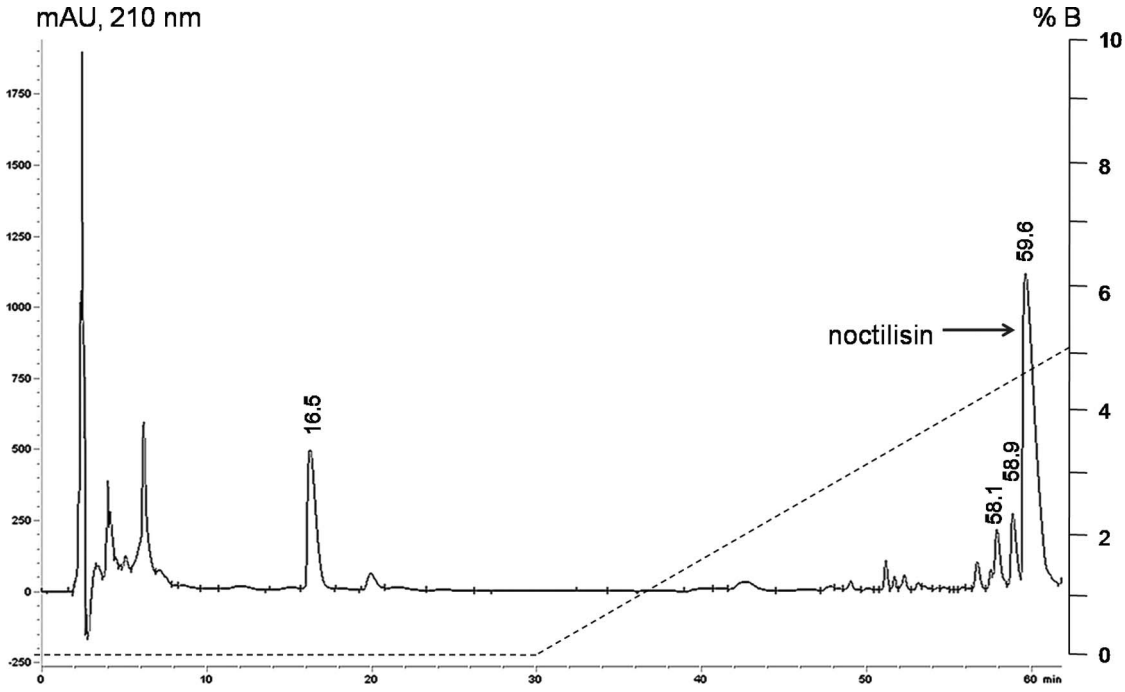


Fig. 3. RP-HPLC purification of noctilisin. Compounds in a 500-μl aliquot of *S. noctilio* venom fraction 3 (F3) were separated using C18 RP-HPLC and following eluent absorbance at 210 nm. Noctilisin activity was limited to a single peak eluting at 59.6 min. Eluent B was 0.04% TFA in acetonitrile.

(Fig. 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 ($\approx$1$\times$ and 10$\times$ saturating values for noctilisin) failed to induce biomarker expression (Fig. 10). Thus, one or both of the O-linked glycans are requisite for noctilisin bioactivity.

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 (Petersen et al. 2011) to contain a secretion signal, as would be expected for a product that must accumulate extracellularly before sequestration in the venom reservoir (Fig. 8). Besides cleavage to remove the inferred signal sequence, the noctilisin polypeptide backbone is posttranslationally 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 (Gorres and Raines 2010). Hydroxyprolyl residues are common in bioactive peptides and are often crucial for activity (Matsubayashi 2011). Examples include Head Peptide I, a 10-amino acid neuropeptide in the mosquito, *Aedes aegypti* (L.), where substitution of proline for hydroxyproline eliminated the peptide's effect on host-seeking behavior (Brown et al. 1994). In plants, hydroxyprolyl residues are critical to the function of several secreted peptide hormones, including CLV3, CLE2, and CEP1 (Matsubayashi 2011). 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 posttranslational 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 (Tian and Ten Hagen 2009). 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 (Hanisch 2001, Hang and Bertozzi 2005). Mucin glycosylation is initiated by attachment of a single α-*N*-acetyl-galactosamine residue to Ser or Thr residues through the action of UDP: GalNAc–polypeptide: GalNAc transferases (Hang and Bertozzi 2005, Bennett et al. 2012). 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 (Ju et al. 2011). In mammals, mucin bio-

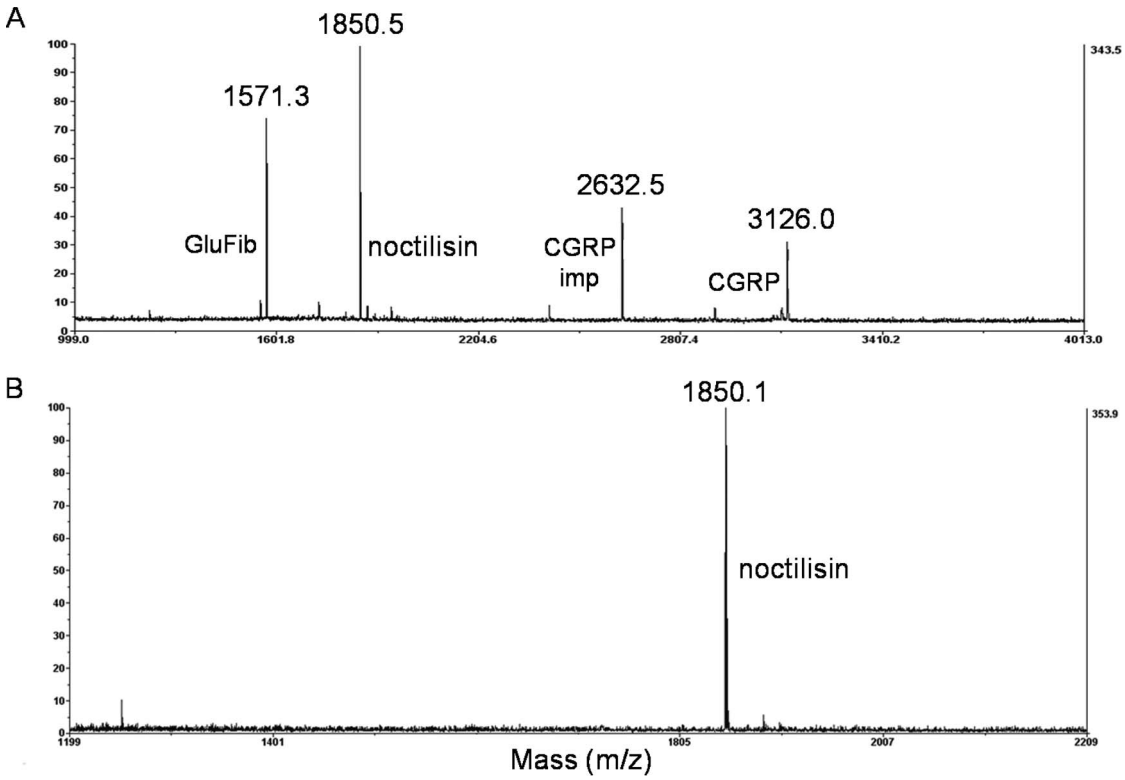


Fig. 4. Molecular mass determination of noctilisin using MALDI-TOF/MS. (A) Depicts the purified glycopeptide along with two internal standards. The m/z 1571 peak is human [Glu]-1-fibrinopeptide B (GluFib, 1570.57 d), and the m/z 3126 peak is calcitonin gene-related peptide Fragments 8–37 (CGRP, 3125.59 d). The additional peak at m/z 2633 in (A) was introduced by the calcitonin gene-related peptide standard (CGRP imp). (B) Shows the glycopeptide alone.

synthesis proceeds through further elaboration of the Tn-antigen structure by attachment of up to 20 additional sugar residues (Grogan et al. 2002), but less extensive structures appear to be the norm for insect mucins (Tian and Ten Hagen 2009). 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 (Ju and Cummings 2005). 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 because plants do not produce Tn-antigen structures that might interfere with immunodetection (Vandenborre et al. 2011b, Yang et al. 2012).

Like antibodies against the Tn-antigen, certain galactose-specific jacalin-related lectins (JRLs) show high binding affinity for the Tn-antigen and as a consequence have been adapted for use as histology reagents to detect its presence (Tachibana et al. 2006, Rougé et al. 2011). Lectins are widely distributed in plants (Raval et al. 2004, Sharon and Lis 2004), where they have long been recognized for their role in plant defense (Michiels et al. 2010, Vandenborre et al. 2011a, Xiang et al. 2011). A JRL with affinity for the Tn-antigen was isolated from ground ivy (*Glechoma hederacea* L.) and shown to have insecticidal properties (Wang et al. 2003). Tn-antigens have been detected previously in mixtures of salivary proteins from certain paper wasp species (Maes et al. 2005). 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 (Birol et al. 2013, Nystedt et al. 2013), 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 apolipophorin III from the hemolymph of the migratory locust, *Locusta migratoria* L. (Hard et al. 1993). The presence of O-glycans modified with phos-

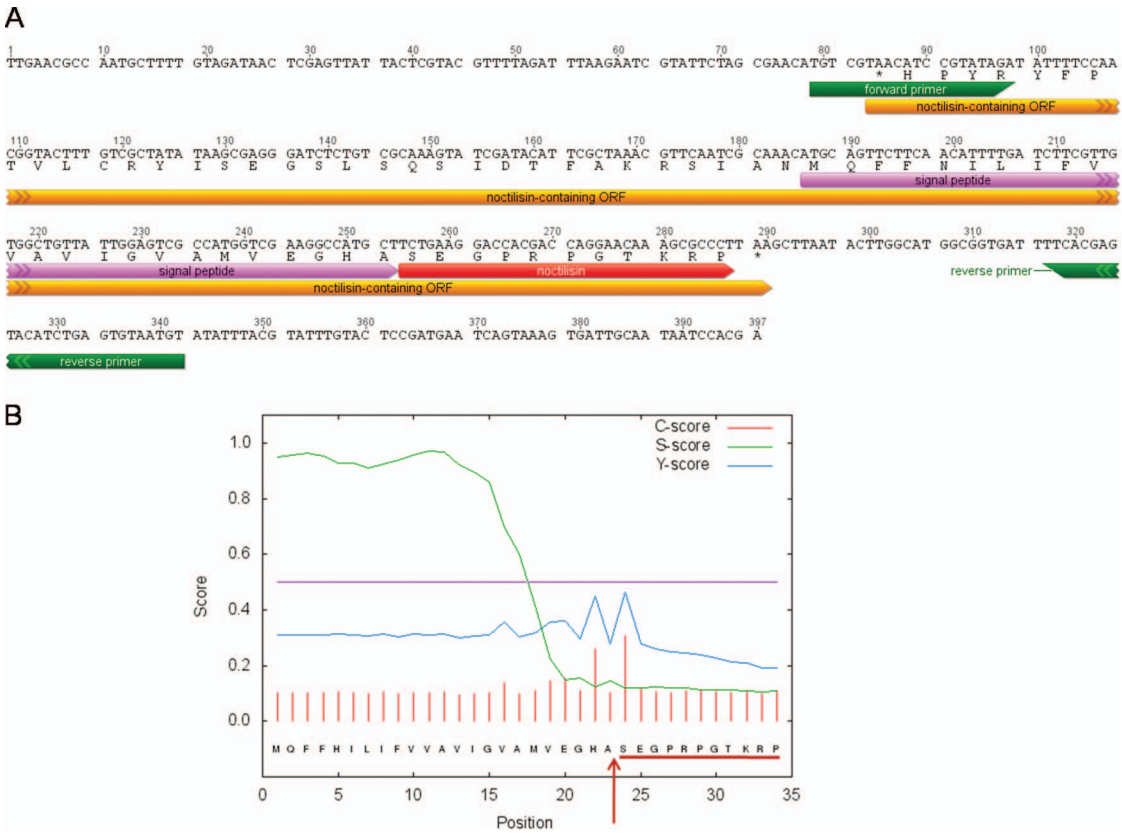
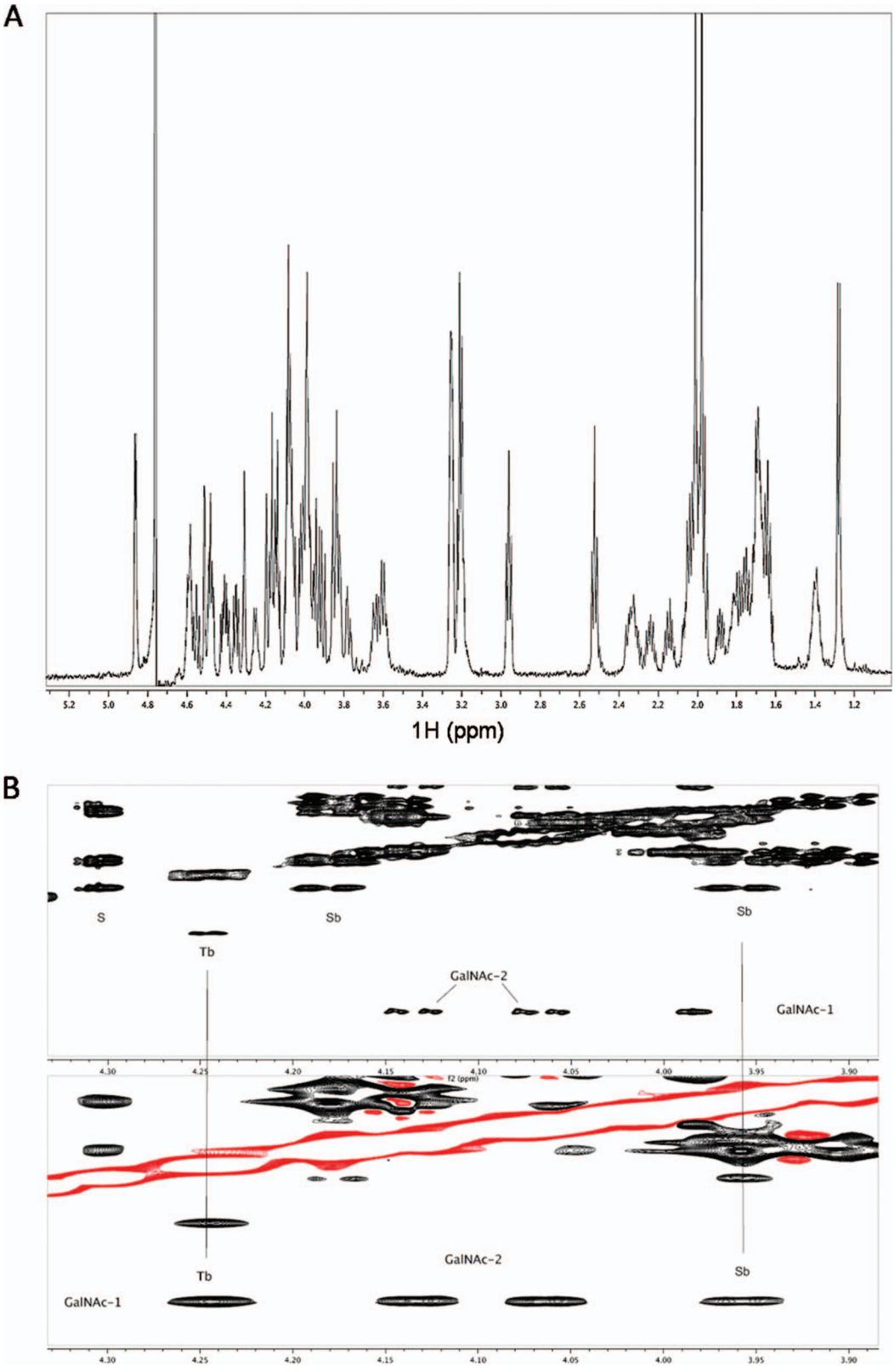


Fig. 5. Noctilisin transcript and amino acid sequence. (A) The signal peptide sequence (pink bar) and the noctilisin peptide sequence (red bar) are contained within the ORF (orange bar). Sanger sequencing of venom gland cDNA amplified a 257-bp portion of the sequence (primers indicated by green bars). Image generated using Geneious, version 6.1 (Biomatters, Auckland, NZ). (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. (Online figure in color.)

phoethanolaminyl groups was reported more recently for salivary proteins from the German yellowjacket, *Vespula germanica* (F.) (Maes et al. 2005). However, similar structures were not detected in salivary proteins from the European hornet, *Vespa crabro* L., (Garenaux et al. 2011), indicating that this modification is not universal across hymenopterans. It will be interesting to test whether phosphoethanolaminyl modification of noctilisin glycosyl groups affects the binding of Tn-specific antibodies and lectins because 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 (Chisholm et al. 2006). Peptide signaling systems involving such receptors have received increasing attention over the past 20 yr for their role in controlling such diverse processes as plant growth and development, self or nonself recognition, and host–pathogen interactions (Germain et al. 2006, Jones and Dangl 2006, Matsubayashi and Sakagami 2006, Katsir et al. 2011, Yamaguchi and Huffaker 2011, Aalen 2013, Albert 2013). Although many peptide signaling systems operate over the short distances between plant cells (Marshall et al. 2011, Murphy et al. 2012), there are peptides that travel long distance through the plant vasculature before eliciting responses (Neumann 2007, Okamoto et al. 2013). For

Fig. 6. (A) NMR spectral analysis of purified noctilisin. Proton spectrum for noctilisin glycopeptide in D_2O at 25°C collected at 600 MHz. (B): 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.(C): 1D (^{1}H)^{31}PHMQC spectrum showing signals from ethanolamine protons as well as GalNAc H6 protons coupled to phosphorous. (Online figure in color.)


A
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1H (ppm)
B
S
Sb
Sb
Tb
GalNAc-2
GalNAc-1
4.30
4.25
4.20
4.15
4.10
4.05
4.00
3.95
3.90
f2 (ppm)
Tb
GalNAc-2
Sb
GalNAc-1

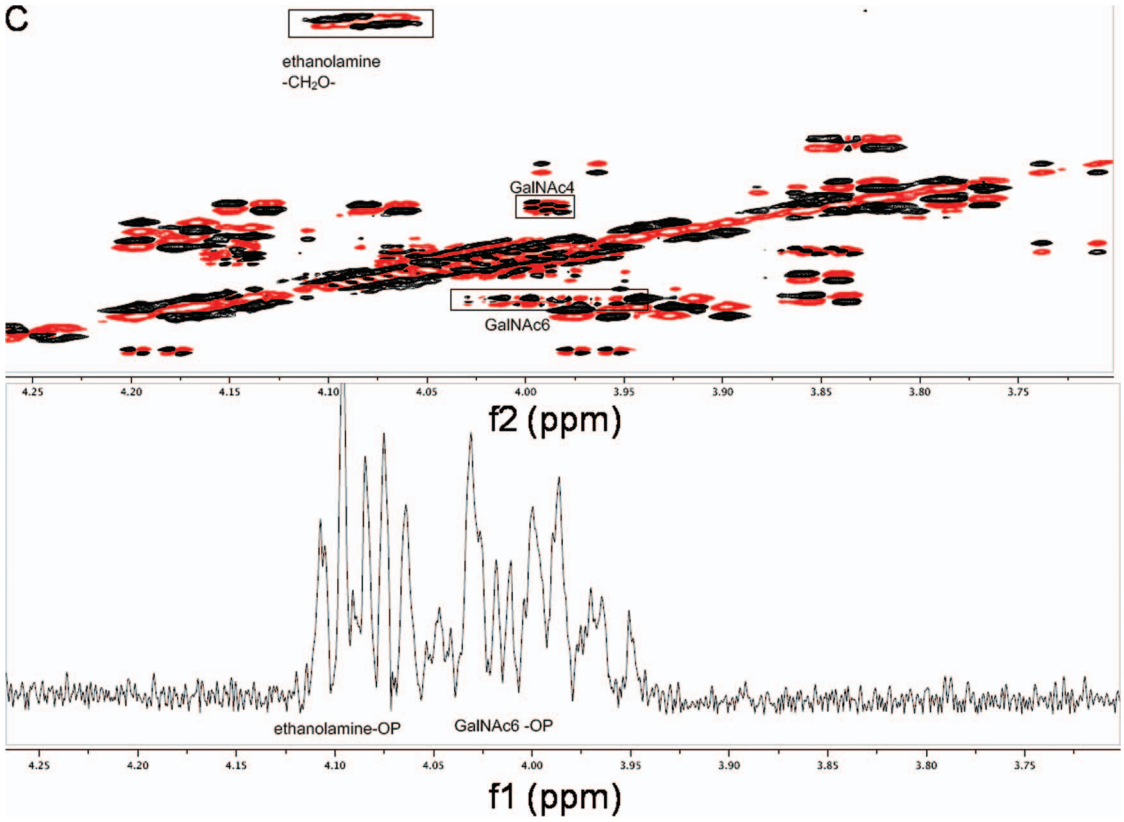


Fig. 6. (Continued).

example, the ethylene-inducing xylanase, originally isolated from *Trichoderma viride* (Dean and Anderson 1991), is translocated through the xylem in tobacco and pepper plants (Bailey et al. 1991) before binding a specific leucine-rich repeat receptor-like protein that undergoes endocytosis and causes a hypersensitive response (Ron and Avni 2004).

Alternative mechanisms for noctilisin action are suggested from comparative venomics (Vetter et al. 2011). 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 (Palma 2006). Many individual bioactive peptides from bee and wasp venoms have

Table 1. Proton chemical shifts for noctilisin glycopeptide in D_2O at 25°C

AA sequence	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				

Values referenced from the singly deuterated water (HDO) peak set to 4.760 ppm.

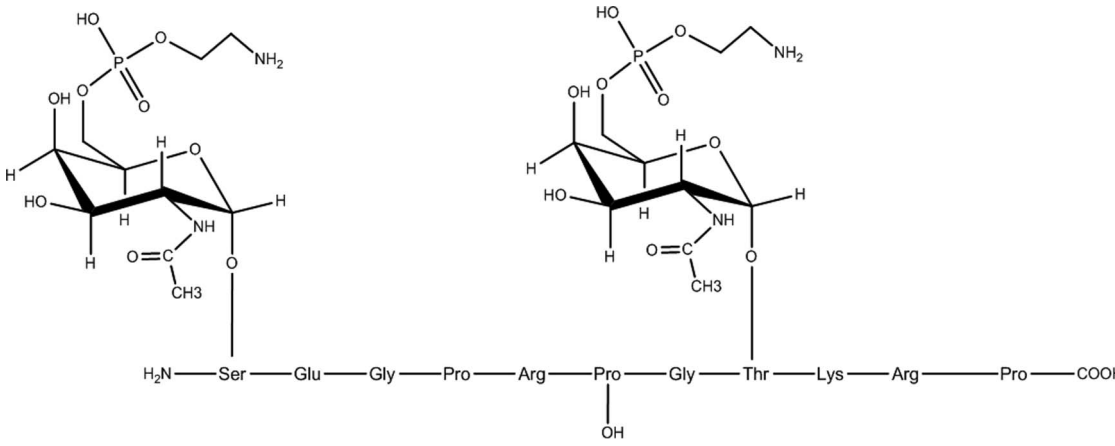


Fig. 7. Noctilisin structure. Residues 1 and 8 of the peptide backbone are O-glycosylated through an α-linkage to *N*-acetylgalactosamine modified by attachment of a phosphoethanolamine moiety to the C6 oxygen. NOE data did not indicate turns or helical regions in the peptide backbone, and identifiable crosspeaks supported an extended structure overall. Figure created using ChemBioDraw software (PerkinElmer, Waltham, MA).

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 ≈50% of the dry weight of honey bee (*Apis mellifera* L.) venom, assumes a bent alpha-helical structure and acts as a chaotropic and cytolytic agent in cell membranes (Raghuraman and Chattopadhyay 2007). Apamin and mast cell-degranulating peptide are closely related 18- and 22-amino acid peptides from honey bee that form ring structures and cause membrane depolarization through interaction with potassium channels (Froy and Gurevitz 1998). Mastoparans are 14-amino acid linear, polycationic peptides common in the venom from social wasps that interact with G-protein receptors and stimulate exocytosis (Higashijima et al. 1988). 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* (F.), is decorated with a single O-linked GalNAc residue, and this glycosylation has been shown to be important for bioactivity (Mackintosh et al. 1998).

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 proline-rich cationic antibacterial peptide (CAP) family was of particular interest (Brogden 2005). Drosocin (which is also related to formaecin I), initially isolated from the he-

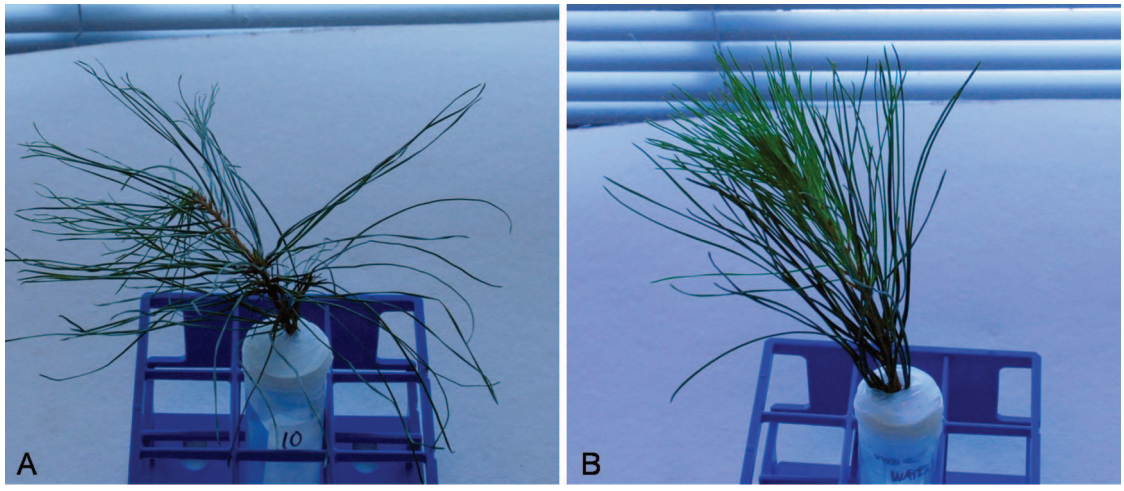


Fig. 8. Typical pine shoot tip bioassay responses. Shoot tips from sensitive *P. radiata* plants were allowed to take up 50 μl of noctilisin in water through their cut ends. After uptake, shoot tips were transferred to water and incubated for 10 d. Cut ends were trimmed every 3 d to minimize xylem blockage. (A) 97 μM purified noctilisin; (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. (Online figure in color.)

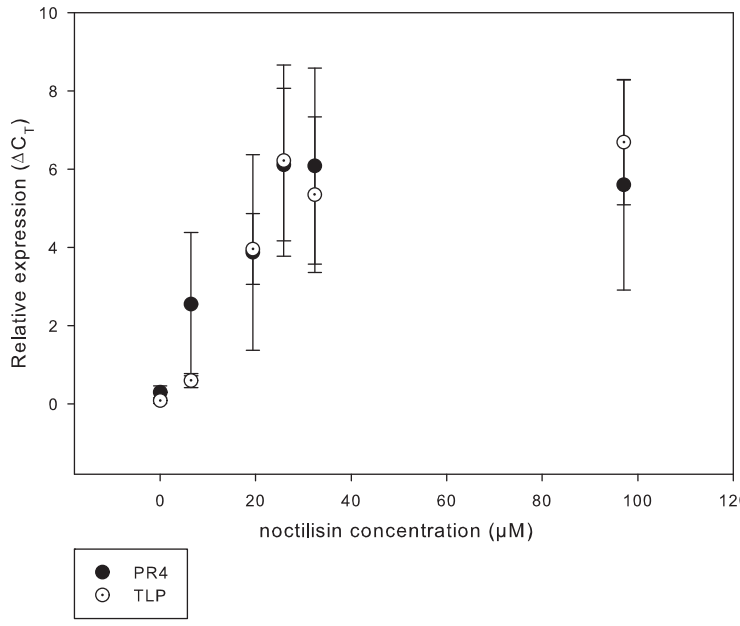


Fig. 9. Dose-dependence of noctilisin bioactivity via qRT-PCR. Shoot tips from two *P. radiata* genotypes with known sensitivity to *S. noctilio* venom were administered 50 μl of noctilisin at the given concentrations in water. Expression levels of the PR4 and TLP biomarker genes were assessed in comparison to the ACT control gene after 24 h. For each data point, $n = 6$ (2 genotypes × 3 shoot tips).

molymph of *Drosophila melanogaster* Meigen, functions as a component of *Drosophila*'s innate immune system (Bulet et al. 1993, Hoffmann and Reichhart 2002, Bennett et al. 2008). 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

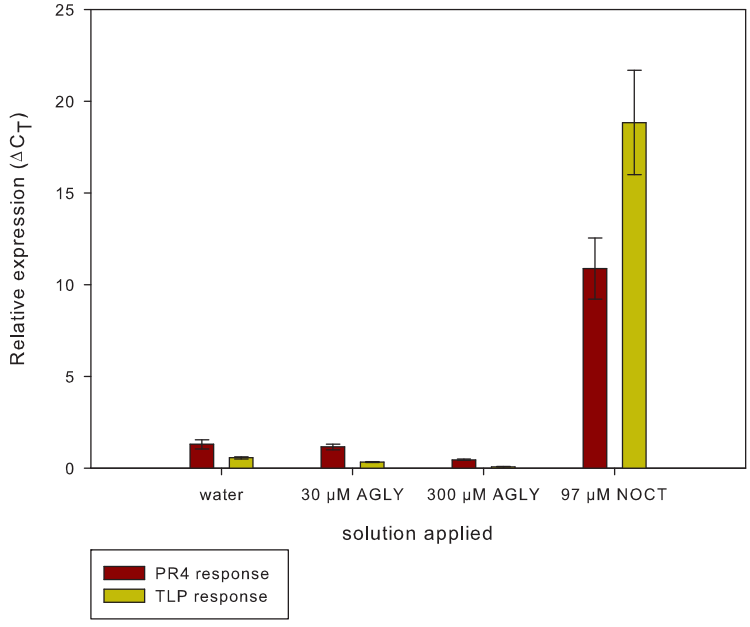


Fig. 10. Dependence via qRT-PCR of noctilisin bioactivity on glycosyl modification. Shoot tips from a single *P. radiata* tree were administered 50 μl of noctilisin (NOCT) or aglyconoctilisin (AGLY) at the given concentrations in water, and then transferred to water. Expression levels of the PR4 and TLP biomarker genes were assessed in comparison with the ACT control gene after 24 h. For each data point, $n = 3$. (Online figure in color.)

Thr11 was described more recently (Levy et al. 2004, Rabel et al. 2004). Differently glycosylated versions of both drosocin and formaecin I were shown to vary in their antibacterial activities (Talat et al. 2011). 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 (Otvos et al. 2000). 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 (Mayer and Bukau 2005, Calloni et al. 2012). 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 have so far shown evidence for binding eukaryotic Hsp70 proteins or cytotoxicity in eukaryotic cells, even when introduced directly into the cytoplasm at high concentrations (Hansen et al. 2012). However, synthetic peptides that bind and inhibit mammalian Hsp70 proteins have been identified and are being tested for their potential as anticancer agents (Rerole et al. 2011). 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 (Kanzaki et al. 2003, Su and Li 2008). 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 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 (Bordeaux et al. 2012). We hope that improved understanding of the system will facilitate breeding of pines to enhance specific resistance against attack from *S. noctilio*.

Acknowledgments

We 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 U.S. Department of Agriculture–National Institute of Food and Agriculture (USDA-NIFA) Graduate Fellowship (2012-67011-19694) awarded to J.M.B., and McIntire–Stennis project GEOZ-0154-MS.

References Cited

Aalen, R. B. 2013. Maturing peptides open for communication. J. Exp. Bot. 64: 5231–5235.

Albert, M. 2013. Peptides as triggers of plant defence. J. Exp. Bot. 64: 5269–5279.

Asgari, S., and D. B. Rivers. 2011. Venom proteins from endoparasitoid wasps and their role in host-parasite interactions, pp. 313–335. *In* M. R. Berenbaum, R. T. Carde and G. E. Robinson (eds.), Annual Review of Entomology, vol. 56. Annual Reviews, Palo Alto, CA.

Bailey, B. A., R. Taylor, J.F.D. Dean, and J. D. Anderson. 1991. Ethylene biosynthesis-inducing endoxylanase is translocated through the xylem of *Nicotiana tabacum* cv *xanthi* plants. Plant Physiol. 97: 1181–1186.

Bennett, C. S., R. J. Payne, K. M. Koeller, and C.-H. Wong. 2008. Biologically Relevant Glycopeptides: Synthesis and Applications, pp. 1795–1857. *In* B. Fraser-Reid, K. Tatsuta and J. Thiem (eds.), Glycoscience. Springer Berlin Heidelberg, Germany.

Bennett, E. P., U. Mandel, H. Clausen, T. A. Gerken, T. A. Fritz, and L. A. Tabak. 2012. Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family. Glycobiology 22: 736–756.

Birol, I., A. Raymond, S. D. Jackman, S. Pleasance, R. Coope, G. A. Taylor, M. M. Saint Yuen, C. I. Keeling, D. Brand, et al. 2013. Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data. Bioinformatics 29: 1492–1497.

Bordeaux, J. M., and J.F.D. Dean. 2012. Susceptibility and response of pines to *Sirex noctilio*, pp. 31–50. *In* B. Slippers, P. de Groot, and M. J. Wingfield (eds.), Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest. Springer, New York, NY.

Bordeaux, J. M., W. W. Lorenz, and J.F.D. Dean. 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.

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, Adelaide, Australia.

Brogden, K. A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3: 238–250.

Brown, M. R., M. J. Klowden, J. W. Crim, L. Young, L. A. Shrouder, and A. O. Lea. 1994. Endogenous regulation of mosquito host-seeking behavior by a neuropeptide. J. Insect Physiol. 40: 399–406.

Bulet, P., J. L. Dimarcq, C. Hetru, M. Lagueux, M. Charlet, G. Hegy, A. Vandorsselaer, and J. A. Hoffmann. 1993. A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution. J. Biol. Chem. 268: 14893–14897.

Calloni, G., T. Chen, S. M. Schermann, H. C. Chang, P. Genevaux, F. Agostini, G. G. Tartaglia, M. Hayer-Hartl, and F. U. Hartl. 2012. DnaK functions as a central hub in the *E. coli* chaperone network. Cell Rep. 1: 251–264.

Chisholm, S. T., G. Coaker, B. Day, and B. J. Staskawicz. 2006. Host-microbe interactions: Shaping the evolution of the plant immune response. Cell 124: 803–814.

Colinet, D., H. Mathe-Hubert, R. Allemand, J. L. Gatti, and M. Poirie. 2013. Variability of venom components in immune suppressive parasitoid wasps: from a phylogenetic to a population approach. J. Insect Physiol. 59: 205–212.

Coutts, M. P. 1969a. Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata. 2. Effects of. S. noctilio* mucus. Aust. J. Biol. Sci. 22: 1153–1161.

Coutts, M. P. 1969b. Mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* .I. Effects of symbiotic fungus *Amylostereum* sp. (Thelophoraceae). Aust. J. Biol. Sci. 22: 915–924.

Coutts, M. P. 1970. The physiological effects of the mucus secretion of *Sirex noctilio* on *Pinus radiata.* Aust. For. Res. 4: 23–26.

Danneels, E. L., D. B. Rivers, and D. C. de Graaf. 2010. Venom proteins of the parasitoid wasp *Nasonia vitripennis:* recent discovery of an untapped pharmacopee. Toxins 2: 494–516.

Dean, J.F.D., and J. D. Anderson. 1991. Ethylene biosynthesis-inducing xylanase 2: purification and physical characterization of the enzyme produced by *Trichoderma viride.* Plant Physiol. 95: 316–323.

Dodds, K. J., and P. de Groot. 2012. Sirex, Surveys and Management: Challenges of having Sirex noctilio in North America, pp. 265–286. *In* B. Slippers, P. de Groot and M. J. Wingfield (eds.), Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest. Springer, New York, NY.

Dodds, K. J., P. de Groot, and D. A. Orwig. 2010. The impact of *Sirex noctilio* in *Pinus resinosa* and *Pinus sylvestris* stands in New York and Ontario. Canadian J. Forest Res. 40: 212–223.

Fong, L. K., and R. K. Crowden. 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.

Froy, O., and M. Gurevitz. 1998. Membrane potential modulators: a thread of scarlet from plants to humans. Faseb J. 12: 1793–1796.

Garenaux, E., E. Maes, S. Levêque, C. Brassart, and Y. Guerardel. 2011. Structural characterization of complex O-linked glycans from insect-derived material. Carbohydr. Res. 346: 1093–1104.

Germain, H., E. Chevalier, and D. P. Matton. 2006. Plant bioactive peptides: an expanding class of signaling molecules. Canadian J. of Botany-Rev. Canadienne De Botanique 84: 1–19.

Gorres, K. L., and R. T. Raines. 2010. Prolyl 4-hydroxylase. Crit. Rev. Biochem. Mol. Biol. 45: 106–124.

Grogan, M. J., M. R. Pratt, L. A. Marcaurelle, and C. R. Bertozzi. 2002. Homogeneous glycopeptides and glycoproteins for biological investigation. Annu. Rev. Biochem. 71: 593–634.

Hang, H. C., and C. R. Bertozzi. 2005. The chemistry and biology of mucin-type O-linked glycosylation. Bioorg. Med. Chem. 13: 5021–5034.

Hanisch, F.-G. 2001. O-glycosylation of the mucin type. Biol. Chem. 382: 143–149.

Hansen, A., I. Schafer, D. Knappe, P. Seibel, and R. Hoffmann. 2012. Intracellular toxicity of proline-rich antimicrobial peptides shuttled into mammalian cells by the cell-penetrating peptide penetratin. Antimicrob. Agents Chemother. 56: 5194–5201.

Hard, K., J. M. Van Doorn, J. E. Thomas-Oates, J. P. Kamerling, and D. J. Van der Horst. 1993. Structure of the Asn-linked oligosaccharides of apolipophorin III from the insect *Locusta migratoria.* Carbohydrate-linked 2-aminoethylphosphonate as a constituent of a glycoprotein. Biochemistry 32: 766–775.

Hennessey, J. P., and W. C. Johnson. 1981. Information content in the circular dichroism of proteins. Biochemistry 20: 1085–1094.

Higashijima, T., S. Uzu, T. Nakajima, and E. M. Ross. 1988. Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G-proteins). J. Biol. Chem. 263: 6491–6494.

Hoffmann, J. A., and J. M. Reichhart. 2002. Drosophila innate immunity: an evolutionary perspective. Nat. Immunol. 3: 121–126.

Jones, J.D.G., and J. L. Dangl. 2006. The plant immune system. Nature 444: 323–329.

Ju, T., and R. D. Cummings. 2005. Protein glycosylation: chaperone mutation in Tn syndrome. Nature 437: 1252.

Ju, T., V. I. Otto, and R. D. Cummings. 2011. The Tn antigen—structural simplicity and biological complexity. Angew. Chem. Int. Ed. 50: 1770–1791.

Kanzaki, H., H. Saitoh, A. Ito, S. Fujisawa, S. Kamoun, S. Katou, H. Yoshioka, and R. Terauchi. 2003. Cytosolic HSP90 and HSP70 are essential components of INF1-mediated hypersensitive response and non-host resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana.* Mol. Plant Pathol. 4: 383–391.

Katsir, L., K. A. Davies, D. C. Bergmann, and T. Laux. 2011. Peptide signaling in plant development. Curr. Biol. 21: R356–R364.

Kingston, R. L., W. A. Baase, and L. S. Gay. 2005. Characterization of nucleocapsid binding by the measles virus and mumps virus phosphoproteins. J. Virol. 79: 10097.

Levy, F., D. Rabel, M. Charlet, P. Bulet, J. A. Hoffmann, and L. Ehret-Sabatier. 2004. Peptidomic and proteomic analyses of the systemic immune response of *Drosophila.* Biochimie 86: 607–616.

Mackintosh, J. A., D. A. Veal, A. J. Beattie, and A. A. Gooley. 1998. Isolation from an ant *Myrmecia gulosa* of two inducible O-glycosylated proline-rich antibacterial peptides. J. Biol. Chem. 273: 6139–6143.

Madden, J. L. 1968. physiological aspects of host tree favourability for the woodwasp, *Sirex noctilio* F. Proc. Ecol. Soc. Aust. 3: 147–149.

Madden, J. L. 1975. Analysis of an outbreak of woodwasp, *Sirex-Noctilio* F (Hymenoptera, Siricidae), in Pinus-Radiata. Bull. Entomol. Res. 65: 491–500.

Maes, E., E. Garénaux, G. Strecker, Y. Leroy, J.-M. Wieruszeski, C. Brassart, and Y. Guérardel. 2005. Major O-glycans from the nest of *Vespula germanica* contain phosphoethanolamine. Carbohydr. Res. 340: 1852–1858.

Marshall, E., L. M. Costa, and J. Gutierrez-Marcos. 2011. Cysteine-rich peptides (CRPs) mediate diverse aspects of cell-cell communication in plant reproduction and development. J. Exp. Bot. 62: 1677–1686.

Matsubayashi, Y. 2011. Post-translational modifications in secreted peptide hormones in plants. Plant Cell Physiol. 52: 5–13.

Matsubayashi, Y., and Y. Sakagami. 2006. Peptide hormones in plants. Annu. Rev. Plant Biol. 57: 649–674.

Mayer, M. P., and B. Bukau. 2005. Hsp70 chaperones: cellular functions and molecular mechanism. Cell. Mol. Life Sci. 62: 670–684.

Michiels, K., E.J.M. Van Damme, and G. Smagghe. 2010. Plant-insect interactions: what can we learn from plant lectins? Arch. Insect Biochem. Physiol. 73: 193–212.

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.

Murphy, E., S. Smith, and I. De Smet. 2012. Small signaling peptides in *Arabidopsis* development: how cells communicate over a short distance. Plant Cell. 24: 3198–3217.

Neumann, P. M. 2007. Evidence for long-distance xylem transport of signal peptide activity from tomato roots. J. Exp. Bot. 58: 2217–2223.

Nystedt, B., N. R. Street, A. Wetterbom, A. Zuccolo, Y. C. Lin, D. G. Scofield, F. Vezzi, N. Delhomme, S. Giacomello, A. Alexeyenko, et al.. 2013. The Norway spruce genome sequence and conifer genome evolution. Nature 497: 579–584.

Okamoto, S., H. Shinohara, T. Mori, Y. Matsubayashi, and M. Kawaguchi. 2013. Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase. (http://www.nature.com/ncomms/2013/130812/ncomms3191/full/ncomms3191.html).

Otvos, L., I. O., M. E. Rogers, P. J. Consolvo, B. A. Condie, S. Lovas, P. Bulet, and M. Blaszczyk-Thurin. 2000. Interaction between heat shock proteins and antimicrobial peptides. Biochemistry 39: 14150–14159.

Palma, M. S. 2006. Insect venom peptides, pp. 389–396. *In* A. J. Kastin (ed.), Handbook of Biologically Active Peptides. Academic, New York, NY.

Petersen, T. N., S. Brunak, G. von Heijne, and H. Nielsen. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8: 785–786.

Piek, T. 1986. Venoms of the Hymenoptera: Biochemical, Pharmacological and Behavioural Aspects. Academic, London, United Kingdom.

Rabel, D., M. Charlet, L. Ehret-Sabatier, L. Cavicchioli, M. Cudic, L. Otvos, and P. Bulet. 2004. Primary structure and in vitro antibacterial properties of the *Drosophila melanogaster* attacin C pro-domain. J. Biol. Chem. 279: 14853–14859.

Raghuraman, H., and A. Chattopadhyay. 2007. Melittin: a membrane-active peptide with diverse functions. Biosci. Rep. 27: 189–223.

Raval, S., S. B. Gowda, D. D. Singh, and N. R. Chandra. 2004. A database analysis of jacalin-like lectins: sequence-structure-function relationships. Glycobiology 14: 1247–1263.

Rerole, A. L., J. Gobbo, A. De Thonel, E. Schmitt, J.P.P. de Barros, A. Hammann, D. Lanneau, E. Fourmaux, O. Deminov, O. Micheau, et al. 2011. Peptides and aptamers targeting HSP70: a novel approach for anticancer chemotherapy. Cancer Res. 71: 484–495.

Ron, M., and A. Avni. 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.

Rougé, P., W. Peumans, E. M. Damme, A. Barre, T. Singh, J. Wu, and A. Wu. 2011. Glycotope structures and intramolecular affinity factors of plant lectins for tn/t antigens, pp. 143–154. *In* A. M. Wu (ed.), The Molecular Immunology of Complex Carbohydrates. Springer, New York, NY.

Rozen, S., and H. J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers, pp. 365–386. *In* K. S. and M. S. (eds.), Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, NJ.

Sharon, N., and H. Lis. 2004. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology 14: 53R–62R.

Slippers, B., P. de Groot, and M. J. Wingfield. 2012. Sirex woodwasp and its fungal symbiont: Research and management of a worldwide invasive pest. Springer, New York, NY.

Su, P. H., and H. M. Li. 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.

Tachibana, K., S. Nakamura, H. Wang, H. Iwasaki, K. Tachibana, K. Maebara, L. Cheng, J. Hirabayashi, and H. Narimatsu. 2006. Elucidation of binding specificity of jacalin toward O-glycosylated peptides: quantitative analysis by frontal affinity chromatography. Glycobiology 16: 46–53.

Talat, S., M. Thiruvikraman, S. Kumari, and K. J. Kaur. 2011. Glycosylated analogs of formaecin I and drosocin exhibit differential pattern of antibacterial activity. Glycoconjugate J. 28: 537–555.

Talbot, P.H.B. 1977. Sirex-Amylostereum-Pinus association. Ann. Rev. Phytopathol. 15: 41–54.

Tian, E., and K. G. Ten Hagen. 2009. Recent insights into the biological roles of mucin-type O-glycosylation. Glycoconjugate J. 26: 325–334.

Vandenborre, G., G. Smagghe, and E.J.M. Van Damme. 2011a. Plant lectins as defense proteins against phytophagous insects. Phytochemistry 72: 1538–1550.

Vandenborre, G., G. Smagghe, B. Ghesquière, G. Menschaert, R. Nagender Rao, K. Gevaert, and E.J.M. Van Damme. 2011b. Diversity in protein glycosylation among insect species. PLoS ONE 6: e16682. (http://dx.doi.org/10.1371%2Fjournal.pone.0016682).

Vetter, I., J. L. Davis, L. D. Rash, R. Anangi, M. Mobli, P. F. Alewood, R. J. Lewis, and G. F. King. 2011. Venomics: a new paradigm for natural products-based drug discovery. Amino Acids 40: 15–28.

Wang, W. F., B. Hause, W. J. Peumans, G. Smagghe, A. Mackie, R. Fraser, and E.J.M. Van Damme. 2003. The Tn antigen-specific lectin from ground ivy is an insecticidal protein with an unusual physiology. Plant Physiol. 132: 1322–1334.

Wellner, D., C. Panneerselvam, and B. L. Horecker. 1990. Sequencing of peptides and proteins with blocked N-terminal amino acids N-acetylserine or N-acetylthreonine. Proc. Natl. Acad. Sci. U.S.A. 87: 1947–1949.

Wong, L. K., and R. K. Crowden. 1976. Preliminary studies on mucus secretion of wood wasp, *Sirex noctilio* F. 1. Physicochemical and biochemical properties. Aust. J. Biol. Sci. 29: 21–32.

Xiang, Y., M. Song, Z. Wei, J. Tong, L. Zhang, L. Xiao, Z. Ma, and Y. Wang. 2011. A jacalin-related lectin-like gene in wheat is a component of the plant defence system. J. Exp. Bot. 62: 5471–5483.

Yamaguchi, Y., and A. Huffaker. 2011. Endogenous peptide elicitors in higher plants. Curr. Opin. Plant Biol. 14: 351–357.

Yang, Z., D. P. Drew, B. Jørgensen, U. Mandel, S. S. Bach, P. Ulvskov, S. B. Levery, E. P. Bennett, H. Clausen, and B. L. Petersen. 2012. Engineering mammalian mucin-type O-glycosylation in plants. J. Biol. Chem. 287: 11911–11923.

Received 10 April 2014; accepted 29 June 2014.