(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 June 2015 (18.06.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/087073 A1

(51) International Patent Classification:
C07K 14/435 (2006.01)

(21) International Application Number:
PCT/GB2014/053663

(22) International Filing Date:
11 December 2014 (11.12.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1321938.1 11 December 2013 (11.12.2013) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2015/087073 A1

(54) **Title**: PESTICIDAL FUSION PROTEIN IMPROVEMENTS

(57) **Abstract**: Methods of increasing the biological activity of toxins. Methods of increasing the biological activity of pesticide toxins through the incorporation of pro- regions into nucleic acid constructs for the production of said toxins.

Pesticidal Fusion Protein Improvements

Field of the Invention

The present invention relates to methods of increasing the biological activity of recombinant toxins. The invention also relates to nucleic acid constructs containing a pro-region and the sequence of a toxin, in particular an arthropod toxin.

Background of the Invention

Against a background of increasing global population, the pressures on food production systems to become more efficient are ever increasing. Pests are still a major constraint on crop production despite progress in crop protection measures. Estimates of the potential losses worldwide for the top six crops vary from 25-80% (40% for potato). Some pests and diseases can be controlled by the application of agrochemicals. However, despite the wide range of pesticides available on the market, plant disease is still a major concern.

In the past the majority of research on developing pesticides focused on the identification of chemical entities which could be used for this purpose. However, these non-target specific pesticides often result in environmental damage and can have a negative impact on non-target species (including animal species) and human health. As a result, European Union legislation has been approved which bans certain chemical compounds from use in pesticides. There has, therefore, been a shift to identifying new types of “biopesticides” that can be used for pest management. Biopesticides are generally considered as naturally occurring substances (biochemical pesticides), microorganisms capable of controlling pests (microbial pesticides) and pesticidal substances produced by plants containing added genetic material (plant-incorporated protectants). It is hoped that this drive towards developing biopesticides will result in more environmentally friendly options for preventing plant disease.

Neuropeptide toxins synthesised as venom by spiders and other arthropods have been the subject of research for development as biopesticides. WO2006/052806, WO2005/025313 and US2007/0066529 describe the use of spider toxin venom peptides for use as a biopesticide and Khan *et al.*, 2006 described expression of spider venom toxin in plants to protect the plants from insect attack. The present inventors have previously shown that ω-ACTX-Hv1a, a toxin derived from the funnel-web spider

Hadroncyhe versuta, when fused to a protein capable of mediating translocation of the fusion protein from the invertebrate gut, such as the snowdrop lectin "carrier" GNA, can function as an effective pesticide against a broad range of pests (WO2012/131302).

Summary of the Invention

The present invention in based in part on studies by the inventors into the effect of inclusion of a pro-region in a construct for expression of a recombinant toxin on the biological activity of said recombinant toxin.

The present investigators wished to determine how the biological activity of recombinant toxin proteins expressed *in vitro* might be further improved. To investigate this, the present inventors analysed the DNA sequences of the genes encoding arthropod toxins. The arthropod toxins utilised in WO2012/131302 are small, cysteine-rich proteins belonging to several superfamilies of protein sequences (which include toxins from organisms other than arthropods). The encoding genes include two sequences that are not present in the final protein product; a predicted N-terminal signal peptide that is removed during translation and a predicted pro-region, between the signal peptide and the final sequence of the protein as isolated (see Figure 1A). Pro-regions are a common feature of small peptide toxins in arthropods and other organisms (Windley *et al.*, 2012). The present inventors have surprisingly found that the inclusion of this predicted pro-region in a construct for expression of a recombinant toxin results in greater biological activity compared to a toxin produced from a construct lacking a pro-region. Furthermore, inclusion of a pro-sequence in a construct for the expression of a toxin which does not naturally contain a pro-sequence in its genomic DNA sequence (for example δ-amaurobitoxin-PI1a) again results in an increased biological activity compared to a toxin produced from a construct lacking a pro-sequence.

Accordingly, in a first aspect of the invention, there is provided a method of increasing the biological activity of a recombinant toxin, the method comprising:
providing a nucleic acid construct comprising: (i) a toxin sequence, or a fragment or variant thereof, linked to (ii) a pro-region, or fragment or variant thereof; and optionally expressing the recombinant toxin.

In a second aspect of the invention, there is provided a nucleic acid construct comprising: (i) a pro-region, or fragment or variant thereof; and (ii) a site adjacent to the pro-region into which a toxin gene sequence, or fragment or variant thereof may be inserted.

In an embodiment in accordance with the second aspect of the invention, the nucleic acid construct further comprises the toxin gene sequence or fragment or variant thereof inserted into the site adjacent to the pro-region.

In a third aspect of the present invention there is provided a host cell comprising the nucleic acid construct according to the second aspect of the invention into which a toxin gene sequence has been inserted, or any embodiment thereof.

In a fourth aspect of the present invention there is provided a method of producing a recombinant toxin with increased biological activity, the method comprising culturing a host cell as defined in the third aspect of the invention under conditions suitable for expression of the recombinant toxin.

The toxin according to the abovementioned aspects of the invention is a pesticide toxin. In preferred embodiments of the invention, the toxin may be derived from arthropods, molluscs or other invertebrates.

In one embodiment of the invention, the toxin is an arthropod toxin. The arthropod toxins of the present invention may include ω-ACTX-Hv1a and κ-ACTX-Hv1c from *Hadronyche versuta*, δ-amaurobitoxin-Pl1a from *Pireneitega luctuosus*, *Segestria florentina* toxins Sfl1-8, *Buthus mesotamulus* toxin ButaIT, theraphotoxin Ec2a from *Eucratoscelus constrictus*, cyrtoautoxin As1a from *Apomastus schlingeri*, sicaritoxin Li1a from *Loxosceles intermedia*, and other similar toxins.

The toxin according to the present invention may comprise a peptide of 20-100 amino acid residues. The toxin may contain multiple cysteine residues forming internal disulphide bonds.

In one embodiment, the toxin is ω-ACTX-Hv1a, or a fragment or variant thereof. The ω-ACTX-Hv1a toxin is known in the art (Fletcher *et* al., 1997). It is a toxin isolated from

the funnel-web spider *Hadroncyhe versuta*. The amino acid sequence of ω-ACTX-Hv1a is known, as is the nucleic acid sequence encoding ω-ACTX-Hv1a. ω-ACTX-Hv1a toxin is a calcium channel antagonist which has previously been shown to block invertebrate but not vertebrate calcium channels. In most circumstances it is desirable to use pesticides which do not have activity against vertebrate animals, so as to avoid deleterious effects on humans or animals.

It has previously been reported that ω-ACTX-Hv1a can be used on its own as a pesticide when applied topically to caterpillars (Khan *et al.*, 2006). However, the authors of the abovementioned document report topical application of the peptide in a solution containing imidazole which is known to be insecticidal in its own right. Moreover, no further evidence for insecticidal activity of the peptide alone has been reported, with other disclosures covering ω-ACTX-Hv1a only stating activity by injections into invertebrate pest animals.

The present inventors have previously shown that the biological activity of recombinant ω-ACTX-Hv1a can be improved by creation of a fusion protein, whereby the toxin ω-ACTX-Hv1a is fused to a "carrier" peptide which can mediate translocation of the fusion protein from the invertebrate gut (WO2012/131302). The inventors used the plant lectin GNA as an example of such a carrier peptide.

To investigate how the biological activity of recombinant toxin fusion proteins might be otherwise improved, the present inventors analysed the DNA sequences of the genes encoding arthropod toxins, in particular ω-ACTX-Hv1a. It was found that many arthropod genes contain a predicted pro-region, not present in the final protein, which had not previously been incorporated into constructs for expressing fusion proteins *in vitro*. As can be seen herein, the inventors incorporated the sequence of the pro-region into a nucleic acid construct for the production of a recombinant toxin protein. The inventors found that a recombinant ω-ACTX-Hv1a produced from a construct containing a pro-region administered to a range of invertebrate pests either by injection or when included in the diet resulted in increased paralysis and mortality compared to producing the recombinant ω-ACTX-Hv1a from an unmodified construct (i.e. containing the ω-ACTX-Hv1a sequence without an additional pro-region). Therefore, ω-ACTX-Hv1a peptide toxin can be very effective as a pesticide to invertebrate animals when supplied in this form.

As used herein, and as further explained below, "pesticide" refers to a chemical substance, biological agent (such as a virus or bacterium), antimicrobial, disinfectant or device used against any pest. Pests include insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes (roundworms), and microbes that destroy property, spread disease or are a vector for disease or cause a nuisance. However, for the present invention by "pesticide" we mean that the pest is any invertebrate animal that destroys property, particularly agricultural commodities.

In an alternative embodiment, the invention may comprise the δ-amaurobitoxin-PI1a toxin, or a fragment or variant thereof.

The toxin δ-amaurobitoxin-PI1a is from the spider *Pireneitega luctuosa* and does not contain a predicted pro-region in its endogenous gene sequence. Surprisingly, the present inventors have found that including a pro-region (designed based on similar sequences present in the global database) in an expression construct for recombinant δ-amaurobitoxin-PI1a results in a recombinant toxin with increased biological activity against invertebrate pests. This unexpected finding demonstrates that the present invention can be used to increase the biological activity of recombinant toxins which do not contain pro-regions associated with their endogenous gene sequences by designing and incorporating pro-regions into expression constructs.

While not wishing to be bound by theory, it is believed that the inclusion of a pro-region in the nucleic acid construct, results in improved folding of the toxin when expressed *in vitro.*

By "fragment or variant" we include that the toxin sequence of the invention can vary from the naturally occurring sequence with the proviso that the fragment or variant substantially retains the biological activity of the toxin. By retain the biological activity of the toxin it is meant that the fragment and/or variant retains at least a portion of the pesticide activity as compared to the native toxin. Typically the fragment and/or variant retains at least 50%, such as 60%, 70%, 80% or 90% activity. In some instances the fragment and/or variant may have a greater pesticide activity than the native toxin. In some embodiments the fragment and/or variant may display an increase in another physiological feature as compared to the native toxin. For example, the fragment

and/or variant may possess a greater half-life *in vitro* and/or *in vivo*, as compared to the native toxin.

By "variants" of a sequence we include insertions, deletions and substitutions, either conservative or non-conservative. In particular we include variants of the nucleotide sequence where such changes do not substantially alter the biological activity of the toxin. A skilled person would know that such sequences can be altered without the loss of biological activity. In particular, single changes in the nucleotide sequence may not result in an altered amino acid sequence following expression of the sequence. Furthermore, if changes in the nucleotide sequence result in the incorporation of an alternative amino acid, but wherein the physio-chemical properties of the respective amino acid(s) are not substantially changed (for example, conservative substitutions such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr), the functionality of the respective toxin should not be affected. Moreover small deletions within non-functional regions of the toxin can also be tolerated and hence are considered "variants" for the purpose of the present invention. "Variants" also include recombinant toxin proteins in which the amino acids have been post-translationally modified, by for example, glycosylation, or disulphide bond formation. The experimental procedures described herein can be readily adopted by the skilled person to determine whether a "variant" can still function as a toxin.

It is preferred if the variant has a sequence which has at least 75%, yet still more preferably at least 80%, in further preference at least 85%, in still further preference at least 90% and most preferably at least 95% or 97% identity with the "naturally occurring" nucleotide sequence of the toxin.

In a preferred embodiment, the present invention relates to arthropod toxins. The arthropod toxins investigated by the present inventors are small, cysteine-rich proteins belonging to several superfamilies of protein sequences. The gene sequences of some of these toxins contain sequences which are not present in the final protein product. These additional sequences include a predicted N-terminal signal peptide which is removed during translation, and a predicted pro-region, between the signal peptide and the final sequence of the protein as isolated (see Figure 1A).

Most cysteine-rich small peptide toxins are initially translated as larger precursors (70–120 amino acids) which contain a conserved N-terminal signal peptide (of approximately 20 amino acids), removed during translation, a pro-region (of approximately 15-60 amino acids) which shows significant sequence conservation within protein families, and a C-terminal toxin-encoding region which gives rise to the mature toxin and is more variable.

Pro-regions have been predicted from cDNA sequences encoding many toxins, and are generally less variable than the mature toxin sequences when compared between members of protein families, although sequence motifs like those found in signal peptides have not been identified. The pro-region is, however, often rich in acidic amino acid residues (Tedford *et al.*, 2004). For example, the amino acid sequence of the pro-region associated with ω-ACTX-Hv1a toxin is:

EDTRADLQGGEAAEKVFRR (see also Figure 1B)

The amino acid sequence of the pro-region associated with Ao1b toxin is:

ISYEEGKELFQKER

Pro-regions can be identified by comparing the sequence determined for a protein isolated from its normal source with the sequence predicted by the gene encoding it. Such a comparison can show whether a proteolysis or cleavage step has taken place co- or post-translationally to obtain the final protein product. Pro-regions are removed from the N-terminus of a mature protein; however, they differ from signal peptides which are involved in routing polypeptides into secretory pathways. Signal peptides can be identified using a software algorithm such as SignalP* (Nielsen *et al.*, 1997) based on the protein sequence predicted by the gene. To identify a pro-region, the directly determined protein sequence is first compared to the predicted sequence to show that a region is removed from the N-terminus; the presence of a signal peptide is then determined by prediction from the software; the pro-region can then be identified as the sequence region between the signal peptide and the mature protein N-terminus. Pro-regions in arthropod toxins can be predicted, based on the concepts outlined above, using software (SpiderP) freely available on the Arachnoserver database (http://www.arachnoserver.org/spiderP.html; Wong *et al.*, 2013). This support vector

machine (SVM) method utilises a specifically designed algorithm to combine both local and global sequence information.

Pro-regions identified using the abovementioned methods can be utilised in the present invention. The pro-region of the abovementioned aspects of the invention may be associated with the toxin of the invention in its naturally occurring sequence. Alternatively, the sequence of a pro-region may be designed based on the sequences available in global databases, or identified based on the abovementioned methods, and incorporated into nucleic acid constructs of toxins which are not associated with pro-regions normally (i.e. in the naturally occurring sequence) or associated with a different pro-region in the naturally occurring sequence.

In an embodiment of the invention, the pro-region comprises the amino acid sequence EDTRADLQGGEAAEKVFRR, or a fragment or variant thereof.

In a further embodiment of the invention, the pro-region comprises the amino acid sequence ISYEEGKELFQKER, or a fragment or variant thereof.

By “fragment or variant” of the pro-region we include that the nucleic acid sequence of the pro-region can differ from that known in the art and that naturally occurring, with the proviso that the fragment or variant substantially retains the biological activity of the pro-region i.e. it is still capable of improving the biological activity of the toxin with which it is associated.

In the accompanying examples, the inventors have shown that the pro-region may be removed during expression, such that it is not present in the final protein or fusion protein. However, it should be appreciated that a protein/fusion protein in which the pro-region is retained following expression still falls within the scope of the present invention.

The nucleic acid constructs according to abovementioned aspects of the invention, and any embodiments thereof, may also contain the sequence of a protein capable of mediating translocation of the protein produced from the construct from the invertebrate gut (a “carrier” protein) or a fragment or variant thereof. Such sequences may be fused to the toxin protein sequence, thereby generating a fusion protein. Any protein which

binds to the insect gut can be used as a carrier protein, providing it is stable under the conditions found in the gut and is non-toxic to mammals.

Suitable proteins capable of functioning as carrier proteins include lectins. Generally, any lectin which binds to the insect gut can be used. In one embodiment of the invention, the carrier proteins are plant lectins.

The inventors have previously shown that certain plant lectins are resistant to gut proteolysis and have the potential to act as carriers to deliver other peptides from the gut to the circulatory system of target species. The present inventors have also shown that fusing plant lectins to a toxin aids translocation across the gut wall of an invertebrate pest, thus increasing biological activity of the toxin, and enabling such a fusion protein to be utilised as a pesticide.

A preferred embodiment of the invention is wherein the carrier protein is a plant lectin selected from any one or more of the following: snowdrop lectin (GNA), garlic lectin *Allium sativum*, pea lectin *Pisum sativum* (P-lec), peanut lectin *Arachis hypogaea*, french bean lectin (PHA, *Phytohaemagglutinin*), or a fragment or variant thereof.

By "fragment or variant" of the plant lectin we include that the nucleic acid sequence of the particular lectin can differ from that known in the art and that naturally occurring, with the proviso that the fragment or variant substantially retains the biological activity of the lectin i.e. it is capable of mediating translocation of the fusion protein from the invertebrate gut.

In a preferred embodiment, the lectin is GNA. The inventors have surprisingly shown in the accompanying examples that a toxin protein produced from a nucleic acid construct containing the sequence of a toxin, in addition to a pro-region and the sequence of GNA, results in an increased biological activity against invertebrate pests compared to the toxin alone, a toxin coupled to GNA only and a toxin coupled to a pro-region only. While the addition of a pro-region, or a fusion to another protein could each be expected to enhance the biological activity of a recombinant toxin, a combination of both modifications would not be expected to produce an additive effect. It was considered that both of these modifications would lead to the same result, namely

correct protein folding, and thus either modification, or both, would lead to the same enhancement of biological activity. The inventors have shown that this is not the case.

As will be appreciated, a starting material for the production of a nucleic acid construct comprising (i) an arthropod toxin sequence, (ii) a pro-region and (iii) a carrier protein sequence is a nucleic acid construct consisting of (i) a pro-region and (ii) a carrier protein sequence.

Accordingly, a fifth aspect of the invention comprises a nucleic acid construct comprising (i) a pro region, or fragment or variant thereof and (ii) a sequence of a protein capable of mediating translocation of a protein produced from the construct from the invertebrate gut (a carrier protein), or a fragment or variant thereof.

The pro-region and carrier protein can be any pro-region/carrier protein discussed in relation to the abovementioned aspects of the invention.

Methods of preparing nucleic acid constructs use routine molecular biology techniques. A variety of methods have been developed to link polynucleotides to form continuous single or double strands, especially double-stranded DNA, for example *via* complementary cohesive termini produced by digestion with restriction enzymes. Suitable methods are described in Sambrook & Russell, Molecular Cloning: A Laboratory Manual: 3rd edition. Such methods can be readily used by the skilled person to prepare a nucleic acid molecule according to the second aspect of the invention. Moreover, the accompanying examples provide further details as to how such molecules are prepared.

A desirable way to prepare the nucleic acid constructs of the invention is to use the polymerase chain reaction. This method may be used for introducing the DNA into a suitable vector, for example by engineering suitable sites for digestion by restriction enzymes, or it may be used to modify the DNA in other useful ways as is known in the art.

In an embodiment of the invention, the nucleic acid constructs according to the abovementioned aspects of the invention, and embodiments thereof, are expression constructs.

An “expression construct” is a term well known in the art. Expression constructs are basic tools for the production of recombinant proteins in biotechnology. The expression construct generally includes a plasmid that is used to introduce a specific nucleic acid sequence into a target cell, a “host cell”. Once the expression construct is inside the cell, protein that is encoded by that nucleic acid sequence is produced by the cellular-transcription and translation machinery ribosomal complexes. The plasmid may also include nucleic acid sequences required for maintenance and propagation of the vector, in some cases through integration into the host genome. The goal of an expression vector is the production of large amounts of stable messenger RNA, and therefore proteins.

The nucleic acid constructs of the invention may further comprise appropriate regulatory sequences, including promoter sequences, terminator fragments, enhancer sequences, marker genes and/or other sequences. For further details see, for example, Sambrook & Russell, Molecular Cloning: A Laboratory Manual: 3rd edition.

The nucleic acid constructs may be further engineered to contain regulatory sequences that act as enhancer and promoter regions and lead to efficient transcription of the fusion protein sequence carried on the construct. Many parts of the regulatory unit are located upstream of the coding sequence of the heterologous gene and are operably linked thereto. The nucleic acid construct may further contain a downstream 3’ untranslated region comprising a polyadenylation site, if expression in a eukaryotic host, for example *Pichia pastoris*, is envisaged. The regulatory sequences can direct constitutive or inducible expression of the heterologous coding sequence.

The methods used to join individual nucleic acid fragments to create the nucleic acid constructs of the invention, may introduce 2-4 extra amino acid residues at the N-terminus of the pro-region, and up to 12 amino acids at the C-terminus of the carrier protein. There may also be short “linker” regions between the toxin and carrier sequences. These additional amino acid residues maintain the coding sequence and do not affect activity of the toxins or fusion proteins.

As used herein, the term "biological activity" refers to the toxicity of the recombinant toxin to invertebrate pests. The calculation of biological activity may be based on the LD_{50}.

The incorporation of a pro-region into a nucleic acid construct for the expression of a recombinant toxin can result in at least a 25%, 50%, 100%, 200%, 300%, 400% or higher increase in the biological activity, compared to the biological activity of a recombinant toxin produced from a nucleic acid construct without a pro-region. In the present case, such a biological activity may be a pesticide activity, which can be measured by a variety of techniques, including pest death, reduced life span, reproduction limitation such as reduced fertility or egg production and the like.

The further incorporation of a sequence of a carrier protein, into an expression construct for the expression of a recombinant toxin can result in at least 25%, 50%, 100%, 200%, 300%, 400% or higher increase in the biological activity, compared to the biological activity of a recombinant toxin produced from a nucleic acid construct containing the toxin sequence and a pro-region.

The expression system of the present invention can be either prokaryotic or eukaryotic. Suitable expression systems include bacterial expression systems (for example *E. coli* and *Bacillus subtilis*), yeast expression systems (for example *Saccharomyces cerevisiae* and *Pichia pastoris*), filamentous fungi expression systems (for example *Aspergillus*), and plant, animal and insect cell expression systems. However, it is preferred that the expression system utilised is the yeast *Pichia pastoris*. *Pichia* protein expression systems are well known in the art, and consequently cells for use as host cells can be readily obtained.

The host cell of the present invention can be prokaryotic or eukaryotic. Preferred prokaryotic host cells are typically strains of *E. coli* such as, for example the *E. coli* strains DH5 and RR1. Preferred eukaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell lines. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. However, it is preferred that the host cell is the yeast *Pichia pastoris*. *Picha* protein expression systems are well known in the art, and hence cells for use as host

cells can be readily obtained. Particularly preferred is where the cell strain is SMD1168H, which can be obtained from Invitrogen™.

Transformation of appropriate cell hosts with a nucleic acid construct is accomplished by well known methods that typically depend on the type of vector used. With regard to the transformation of prokaryotic host cells, see for example, Sambrook & Russell, Molecular Cloning: A Laboratory Manual: 3rd edition. Transformation of yeast cells is described in Sherman *et al.*, 1986.

Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cells, bacterial cells, insect cells and vertebrate cells. Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente, 1990.

Successfully transformed cells, i.e. cells that contain a nucleic acid construct according to the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of a nucleic acid construct of the present invention can be grown to produce a toxin fusion protein. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA such as that described by Southern, 1975 or Berent *et al.,* 1985.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal culture, or a culture derived from a monoclonal culture, in a nutrient medium.

An embodiment of the abovementioned aspects of the invention is wherein the nucleic acid construct further comprises a sequence which encodes an affinity tag to aid recovery and purification of the toxin protein, once expressed.

The use of short amino acid tag sequences to aid the affinity purification of recombinant proteins is well known in the art. Indeed, many commercially available protein expression constructs include nucleic acid sequences encoding such tags. The protein of interest is inserted in to the expression construct in such a manner that the affinity tag is linked to said protein. A variety of different affinity tags are known in the

art, including chitin binding protein (CBP), maltose binding protein (MBP), glutathione-S-transferase (GST), and the polyhistidine-tag (His-tag).

A His-tag is an amino acid motif in proteins that consists of at least five histidine (His) residues, often at the N- or C-terminus of the protein. It is also known as hexa histidine-tag, 6xHis-tag, and by the trademarked name His-tag®. They are a well known affinity tag and methods of introducing His-tags to recombinant proteins are known in art, as are routine methods of purifying proteins with His-tags. A preferred embodiment of the invention is wherein the additional affinity tag sequence encodes a His-tag.

The method of the fourth aspect of the invention may further comprise culturing the host cell as described in the third aspect of the invention (and any embodiment thereof described in this specification) for a sufficient time and under appropriate conditions in a culture medium so as to obtain expression of the fusion protein.

Methods of cultivating host cells and isolating recombinant proteins are well known in the art. Examples of suitable purification techniques are described in the accompanying examples. As described above, the fusion protein may comprise an affinity tag so as to aid purification using affinity reagents, as will be know to those skilled in the art.

The recombinant toxin protein with increased biological activity according to the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatograph, hydroxylapatite chromatography and lectin chromatography.

Alternatively the recombinant toxin protein with increased biological activity according to the present invention may be recovered from the supernatant. In this case, the host cell is removed from the supernatant by simple centrifugation as would be appreciated by a person skilled in the art. The protein can be isolated from the culture medium using standard techniques known in the art such as the abovementioned techniques.

The inventors have determined that the recombinant toxin protein with increased biological activity according to the present invention can be used as a pesticide.

A sixth aspect of the invention provides a pesticide composition comprising a toxin protein produced according to the first or fourth aspects of the invention and any embodiment thereof described in this specification.

A pesticide may be a chemical substance, biological agent (such as a virus or bacterium), antimicrobial, disinfectant or device used against any pest. Pests include insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes (roundworms), and microbes that destroy property, spread disease or are a vector for disease or cause a nuisance. However, for the present invention by "pesticide" we mean that the pest is any invertebrate animal that destroys property, particularly agricultural commodities.

More preferably still the toxin protein is capable of destroying, or at least debilitating, insect pests from the following orders: Coleopterans e.g. Southern corn rootworm (*Diabrotica undecimpunctata*); cowpea bruchid (*Callosobruchus maculatus*); Lepidopterans e.g. European cornborer (*Ostinia nubilalis*); tobacco hornworm (*Manduca sexta*); stem borer *(Chilo partellus*): Homopteran pests e.g. Rice brown plant hopper (*Nilaparvata lugens*); rice green leaf hopper (*Nephotettix cinciteps*); potato leaf hopper (*Empoasca fabae*); peach potato aphid (*Myzus persicae*); pea aphid (*Acyrthosiphon pisum*); Dipteran e.g. gout fly *Chlorop pumilionis;* Orthoptera e.g. crickets and locusts; Isoptera e.g. termites; Thysanoptera e.g. thrips; Hymenoptera e.g. ants and arthropod pests of the order Acarina (mites).

Particularly preferred pests include the Lepidopteran *Mamestra brassicae*, Colorado potato beetle (*Leptinotarsa decemlineata*, a Coleopteran), Wheat bulb fly (*Delia coarctata*, an Anthomyiidae) and the cereal aphid *Sitobion avenae*, a Homopteran.

The inventors have also investigated whether recombinant toxin proteins with increased biological activity produced according to the methods of the present invention have pesticidal activity against molluscs. As demonstrated in the accompanying examples, they have surprisingly found that the grey field slug (*Decoceras reticulatum*, a mollusc) is susceptible to the pesticidal activity of a recombinant toxin protein produced according to the present invention. Accordingly, a recombinant toxin protein according to the present invention is capable of destroying,

or at least debilitating, molluscs, including slugs and snails, and particularly grey field slugs.

Preferably the pesticidal composition according to the invention is in the form of any desired formulation such as a solution, emulsion, spray, suspension, powder, foam, paste, granule, aerosol, capsule or other finely or coarsely divided material or impregnant for natural or synthetic material.

In a preferred embodiment the pesticidal composition is in the form of a spray, suspension or the like, in admixture with suitable diluents, adjuvents, preservatives, dispersants, solvents, emulsifying agents or the like. Suitable composition components are those conventionally employed in the art, and in particular being suited to the present oral administration application. The composition may be obtained with use of any suitable solvents, preferably water, alcohol, mineral oil or the like, any suitable solid carriers such as kaolin, clay, talc, chalk, quartz, attapulgite, montmorillonite, diatomaceous earth, silica, or the like, with use of any solid carriers as supports for granules such as calcite, marble, pumice and crushed natural fibre material or the like.

Compositions for use in the invention may additionally be employed in intimate or physical admixture together with other known insecticides, growth promoting or regulating substances, herbicides, fungicides, synergistic agents and the like.

The composition is preferably suitable for physically or chemically associating with plants or their locus and for oral uptake by pathogens.

The composition may therefore comprise a fusion protein (toxin) in an amount of between 0.001% and 99% by weight preferably between 0.5% and 98% by weight, more preferably between 1.0% and 95% by weight.

The term “locus” as used above refers to the physical location where the crop or plant is growing. For example, for agricultural crops, the locus may be a field; for vegetable crops, the locus may be a flowerbed or vegetable patch; and for ornamental plants, the locus may be a flower pot or container.

A seventh aspect of the present invention provides a process for the preparation of a pesticide composition according to the sixth aspect of the invention (and any embodiment thereof described in this specification) which comprises admixture of an amount of toxin protein with increased biological activity produced according to the present invention (and any embodiment thereof described in this specification) with one or more suitable carriers, diluents, adjuvants, preservatives, dispersants, solvents, emulsifying agents in effective pesticidal amount.

An eighth aspect of the present invention provides a method of preventing or treating a pest infection of a plant comprising applying a quantity of the toxin protein with increased biological activity produced according to the present invention or a pesticide composition according to the sixth aspect of the invention (and any embodiment thereof described in this specification) to the plant or its locus of growth; or introducing to the plant the nucleic acid construct according to the present invention.

A range of different mollusc pests can be controlled using the method of the eighth aspect of the invention, particularly the grey field slug (*Decoceras reticulatum*). Accordingly, the method of the eighth aspect of the invention includes where the mollusc is a slug or snail, and particularly a grey field slug.

A ninth aspect of the present invention provides a method of preventing or treating a mollusc pest infection of a plant comprising applying a quantity of toxin protein with increased biological activity produced according to the present invention, or a pesticide composition according to the sixth aspect of the invention (and any embodiment thereof described in this specification) to the plant or its locus of growth, or introducing to the plant a nucleic acid construct according to the present invention.

The toxin protein with increased biological activity produced according to the present invention and the related aspects and embodiments of the invention listed above, in particular the pesticide compositions, can be used as molluscicide.

It will be appreciated that the molluscicide are suitably prepared and formulated so as to allow easy use by the consumer. For example, the molluscicide may be prepared as a liquid which can be sprayed on a crop, or as granules that can also be applied to crops and/or locus.

It is well known in the art that molluscicides are commonly presented in the form of bait (or pellets). When presented in such a format, the user can easily apply the molluscicde to the plant or its locus of growth and so prevent or treat mollusc pest infection.

A tenth aspect of the invention therefore provides a molluscicide bait composition comprising a toxin protein with increased biological activity produced according to the present invention and/or a pesticide composition according to the sixth aspect of the invention.

The pellet or bait can also include a mollusc attractant so as to encourage exposure of the pest to the molluscicide. A mollusc attractant is anything that attracts molluscs. The attractant may be a phagostimulant. Phagostimulants are conventionally used in slug and snail bait formulations to attract gastropods to ingest the molluscicide, and are typically attractants and/or food. Mixtures of phagostimulants with other suitable organic and/or inorganic carriers may also be used. Suitable phagostimulants for molluscicides include ground cereals (such as wheat flour, barley flour, rye flour and rice starch), crushed soya beans, fish meal, molasses, crushed rapeseed and the like. Mixtures of phagostimulants may also be used in the present invention. Other known attractants include beer, yeast and extract of dead slugs. The bait composition may also comprise one or more bird repellents, such as anthraquinone.

The composition may be formulated to provide a slow or delayed release of molluscicide over time, so as to provide long-term protection against molluscs. Suitable slow-release auxiliaries which may be employed in the formulation include, for example, resins (such as urea/formaldehyde resins), soyabean meal, waxes, stearates and oils (such as castor oil).

Other auxiliaries that may be used in the bait or pellet composition of the present invention include, for example, binders (such a methylcelloslove, polyvinylpyrrolidone, polyvinyl alcohol, polyacrylates, polymethacrylates, natural waxes, chemically modified waxes and synthetic waxes, sugars, starch, alginates, agar, lignosulphonates and gum Arabic), humectants (such as polyalcohols, for example sugars or glycerol), preservatives, colorants and repellents for warm-blooded species.

The bait composition may also be coated to protect it from moisture degradation. Such a coating may extend the life of the bait composition, and reduce the re-application frequency needed. Suitably the bait composition does not prematurely degrade when it is applied to damp soil.

The bait composition is typically provided in the form of granules or pellets. The size of the pellets is such that they can be readily consumed by the target gastropods to ensure ingestion. Typically, the pellets are from about 1 mm to about 5 mm in length.

An eleventh aspect of the invention provides a transgenic plant or progeny thereof comprising a nucleic acid construct according to the present invention capable of expressing a toxin in accordance with the present invention.

By "transgenic plant" we include that the plant may have a nucleic acid construct according to the present invention incorporated into its germline or that the plant may contain an exogenous nucleic acid construct according to the present invention, either of which can be expressed in the plant.

It will be appreciated that a transgenic plant containing a nucleic acid construct according to the invention, when regulated in the correct manner, will produce a toxin protein with increased biological activity according to a method of the invention. The protein/fusion protein produced will function as a pesticide.

A range of different plant species can be modified to include the nucleic acid construct according to the present invention.

A person skilled in the art would know that any monocot or dicot plant can be used. A dicot plant may be selected from the families including, but not limited to Asteraceae, Brassicaceae (e.g. *Brassica napus*), Chenopodiaceae, Cucurbitaceae, Leguminosae (Caesalpiniaceae, Aesalpiniaceae Mimosaceae, Papilionaceae or Fabaceae), Malvaceae, Rosaceae or Solanaceae For example, the plant may be selected from lettuce, sunflower, Arabidopsis, broccoli, spinach, water melon, squash, cabbage, tomato, potato, capsicum, tobacco, cotton, okra, apple, rose, strawberry, alfalfa, bean,

soybean, field (fava) bean, pea, lentil, peanut, chickpea, apricots, pears, peach, grape vine or citrus species.

Also included are biofuel and bioenergy crops such as sugar cane, oilseed rape/canola, linseed, willow, poplar, poplar hybrids, switchgrass, Miscanthus or gymnosperms, such as loblolly pine. Also included are crops for silage (e.g. forage maize), grazing or fodder (e.g. grasses, clover, sanfoin, alfalfa), fibres (e.g. cotton, flax), building materials (e.g. pine, oak), pulping (e.g. poplar), feeder stocks for the chemical industry (e.g. high erucic acid oil seed rape, linseed).

A monocot plant may, for example, be selected from the families Arecaceae, Amaryllidaceae or Poaceae. For example, the plant may be a cereal crop, such as wheat, rice, barley, maize, oat, sorghum, rye, onion, leek, millet, buckwheat, turf grass, Italian rye grass, switchgrass, Miscanthus, sugarcane or Festuca species.

Preferably, the plant is a crop plant. By crop plant is meant any plant which is grown on a commercial scale for human or animal consumption or use or other non-food/feed use. Preferred plants are maize, tobacco, wheat, rice, oilseed rape, sorghum, soybean, potato, tomato, barley, pea, bean, field bean, cotton, lettuce, broccoli or other vegetable brassicas.

The nucleic acid construct can be introduced as a transgene into the plant. This can be carried out by various methods as known in the field of plant genetic engineering, for example using transformation with Agrobacterium, particle bombardment, electroporation or viral transformation. Such techniques are well known in the art. The use of specific techniques for each of the plant species listed herein are also well known. The methods of the art can be readily adopted by the skilled person to prepare a transgenic plant or progeny thereof comprising a nucleic acid sequence according to the invention.

When used for preparing a transgenic plant of this aspect of the invention, the nucleic acid construct is typically placed within an “expression cassette” suitable for arranging the expression of the nucleic acid sequence in the plant. The expression cassette will typically contain nucleic acid sequence which act to regulate the expression of the nucleic acid in the plant, e.g. a promoter region.

Some promoters can drive constitutive expression of the nucleic acid in plants, including the well known 35S promoter, 19S promoter or the ubiquitin promoter.

Other promoters can be used to regulate organ or tissue specific expression of the nucleic acid construct. A "tissue-specific promoter" or "tissue-preferred promoter" refers to a regulated promoter that is not expressed in all plant cells but only in one or more cell types in specific organs (such as leaves or seeds), specific tissues (such as embryo or cotyledon), or specific cell types (such as leaf parenchyma or seed storage cells). These also include promoters that are temporally regulated, such as in early or late embryogenesis, during fruit ripening in developing seeds or fruit, in fully differentiated leaf, or at the onset of sequence. Suitable promoters include the napin-gene promoter from rapeseed, the USP-promoter from *Vicia faba*, the oleosin-promoter from *Arabidopsis*, the phaseolin-promoter from *Phaseolus vulgaris*, the Bce4-promoter from Brassica or the legumin B4 promoter as well as promoters conferring seed specific expression in monocot plants.

Promoters active in photosynthetic tissue in order to drive transcription in green tissues such as leaves and stems include ribulose-l,5-bisphosphate carboxylase (RbcS) promoters such as the RbcS promoter from eastern larch (*Larix laricina*), the pine cab6 promoter, the Cab-1 gene promoter from wheat, the CAB-1 promoter from spinach, the cablR promoter from rice, the pyruvate orthophosphate dikinase (PPDK) promoter from corn, the *Arabidopsis thaliana* SUC2 sucrose-H+ symporter promoter. Nucleic acid sequences representative of such promoters can be readily identified by the skilled person from, for example, GenBank.

Promoters suitable for preferential expression in plant root tissues include, for example, the promoter derived from corn nicotianamine synthase gene and rice RCC3 promoter.

Promoters suitable for preferential expression in plant vascular tissues include, for example, rice sucrose synthase-1 gene (RSs1).

Inducible promoters include promoters which are responsive to abiotic and biotic environmental stimuli. Abiotic environmental stimuli include light, temperature and water availability. Biotic environmental stimuli include pathogens, (including viral

induced, bacterial induced, fungal induced, insect induced and nematode induced promoters), interactions with symbionts and herbivores. Promoters may also be responsive to movement, touch, tissue damage and phytohormones (including abscissic acid, cytokinins, auxins, giberellins, ethylene, brassinosteroids and peptides such as systemin and nodulation factors). Temporally regulated promoters include circadian regulated promoters as well as those which respond to non-circadian time-keeping mechanisms.

Chemically inducible promoters are especially suitable if gene expression is wanted to occur in a time specific manner. Examples of such promoters are a salicylic acid inducible promoter, a tetracycline inducible promoter, and an ethanol inducible promoter (WO 93/21334).

In order to obtain improved expression in plants, the codon usage of the nucleic acid construct can be modified to form an equivalent, modified or artificial gene or gene part in accordance with techniques, so as to increase the efficiency of expression of the toxin protein in plant cells. Moreover, the nucleic acid may also be inserted in the plastid (e.g. chloroplast) or mitochondrial genome of a plant and expressed there using a suitable promoter. For obtaining enhanced expression in monocot plants such as corn or rice, an intron (e.g. a monocot intron) can also be added to the chimeric gene.

It may be preferred that chimeric nucleic acids of the invention (and suitable for use in the methods of the invention) further comprise nucleic acid sequences for the expression of products that may aid in the identification of plant cells into which the chimeric nucleic acid sequences have been successfully incorporated. Examples of suitable further nucleic acid sequences that may be used in this manner will be apparent to those skilled in the art, and include nucleic acids giving rise to products that confer resistance to substances that may be used for selection (such as antibiotics) or markers that give rise to a detectable product that may be used as the basis for selection (such as a chromogenic enzyme product).

A further aspect the present invention provides a plant transformed with a nucleic acid construct according to the present invention.

In a further aspect the present invention provides a plant seed comprising a nucleic acid construct according to the present invention.

A further aspect of the invention provides the use of a nucleic acid construct according to the present invention or a toxin protein produced according to the present invention in the manufacture of a pesticide or a transgenic plant cell or plant.

A further aspect of the invention provides the use of a pesticide according to the sixth aspect of the present invention (and any embodiment thereof described in this specification) to destroy, or debilitate one or more pests.

A further aspect of the invention provides a nucleic acid construct, toxin protein, composition, vector, host cell, transgenic plant, or methods for the preparation or use thereof substantially as herein described in the description or sequences or illustrated in the Figures.

Detailed Description

The present invention will now be described with reference to the following non-limiting examples and figures, which show:

Figure 1: Schematic of gene structure of toxins containing pro-regions

(A) Schematic of gene structure of toxin proteins containing pro-regions. (B) Sequence of spider toxin, Hv1a. Boxed amino acid sequence corresponds to pro-region.

Figure 2: Expression and purification of recombinant Hv1a and pro-Hv1a toxin

(A) SDS-PAGE gel (20% acrylamide) analysis showing purification of recombinant Strep-tagged Hv1a toxin from culture supernatant. Lanes 1 & 2 are GNA standards (0.5 and 0.25 µg, respectively) and lanes 3 & 4 are peak fractions (10 µl) following elution from Streptactin column with 2.5 mM desthiobiotin. (B) Tris-Tricine gel (15% acrylamide) analysis of recombinant pro-Hv1a, lanes 1 & 2 are peak fractions (10 µl) following elution from a nickel affinity column with 0.2 M imidazole. Arrow depicts major protein product predicted to be pro-Hv1a from which the histidine tag has been cleaved. (C) Western blot analysis of sample in (B) using anti-His antibodies.

Figure 3: SDS-PAGE gel analysis of lyophilised samples of purified pro-Hv1a/GNA, Hv1a/GNA and MODHv1a/GNA

SDS-PAGE analysis (17.5% acrylamide gel) of purified recombinant Hv1a containing fusion protein, gel stained for total proteins with Coomassie Blue. Loading as follows: Lane 1: pro-Hv1a/GNA; Lane 2: Hv1a/GNA; Lane 3: MODHv1a/GNA; Lanes 4-6: GNA standards of 1, 2 and 4 µg, respectively; Lanes 7-9: 12.5, 25 and 50 µg lyophilised purified pro-Hv1a/GNA (to enable quantification of fusion protein content).

Figure 4: Injection toxicity of recombinant pro-Hv1a, pro-Hv1a/GNA and Hv1a/GNA to *Mamestra brassicae*

Percentage survival of 3rd-5th instar *Mamestra brassicae* larvae following the injection of different doses of recombinant pro-Hv1a, pro-Hv1a/GNA or Hv1a/GNA. (A) Percentage survival of 5th instar larvae following injection of various doses of pro-Hva1. (B) Percentage survival of 3rd-4th instar larvae following injection of various doses of pro-Hv1a/GNA (Dose A) or Hv1a/GNA (Dose B). (C) Percentage survival of 5th instar larvae following injection of various doses of pro-Hv1a/GNA (Dose A) and Hv1a/GNA (Dose B).

Figure 5: Ingestion toxicity of recombinant pro-Hv1a, Hv1a/GNA, pro-Hv1a/GNA and GNA to *Mamestra brassicae*

Percentage survival of 3rd instar *Mamestra brassicae* larvae following ingestion of a single 2 µl droplet containing 20 µg of purified pro-Hv1a, Hv1a/GNA, pro-Hv1a/GNA or GNA. Control larvae were fed on a droplet containing no added protein (n=10 per treatment).

Figure 6: Ingestion toxicity of recombinant pro-Hv1a, pro-Hv1a/GNA, Hv1a/GNA or GNA to *Acyrthosiphon pisum* and *Sitobion avenae*

Percentage survival of (A) *Acyrthosiphon pisum* (pea aphids) and (B) *Sitobion avenae* (cereal aphids) with artificial diets containing 0.05 – 0.75 mg/ml purified recombinant pro-Hv1a, pro-Hv1a/GNA, Hv1a/GNA or GNA.

Figure 7: Injection toxicity of recombinant pro-Hv1a/GNA and Hv1a/GNA to *Deroceras reticulatum*

Percentage survival of *Deroceras reticulatum* (200 mg ± 40 mg) injected with 100, 50 or 25 µg of Hv1a/GNA or pro-Hv1a/GNA. n=18 for control treatment; n=10 for 100 µg dose; and n=8 for 50 and 25 µg doses.

Figure 8: Expression and purification of recombinant PI1a and Ao1bPro-PI1a toxin

(A) PI1a toxin derived from a construct encoding the mature toxin sequence separated on "normal" SDS-PAGE; M indicates marker, loadings of PI1a are 5 and 10 µg. (B) PI1a toxin (5 µg) separated on SDS-PAGE after denaturation by 6 M urea. (C) Recombinant PI1a toxin derived from a construct containing the pro-region designated Ao1b on SDS-PAGE, loadings of Ao1bPro-PI1a are 2.5 µg. (D) Western blotting of purified Ao1bPro-PI1a (25, 50 & 100 ng) using anti-His antibodies.

Figure 9: Characterisation of purified recombinant PI1a/GNA fusion proteins by SDS-PAGE

(A) SDS-PAGE analysis of PI1a/GNA fusion protein (10 µg) and GNA standard (5 µg). (B) Deglycosylation of PI1a/GNA fusion protein using PNGase F (band indicated by open arrowhead), GNA standard (5 µg). (C) SDS-PAGE analysis of Ao1bPro-PI1a/GNA (1, 2 and 4 µg), GNA standard (5 µg). (D) SDS-PAGE analysis of Hv1aPro-PI1a/GNA (1, 2 and 4 µg), GNA standards (1, 2 and 4 µg).

Figure 10: Injection toxicity of recombinant PI1a and Ao1bPro-PI1a to *Mamestra brassicae*

(A) Percentage survival of 5th instar *Mamestra brassicae* larvae following injection of different doses of purified recombinant PI1a. (B) Percentage survival of 5th instar *Mamestra brassicae* larvae following injection of different doses of purified recombinant PI1a (Dose A) or Ao1bPro-PI1a (Dose B).

Figure 11: Injection toxicity of recombinant PI1a/GNA, Ao1bPro-PI1a/GNA and Pro-HV1a-PI1a/GNA to *Mamestra brassicae*

Percentage survival of 5th instar *Mamestra brassicae* larvae following injection of different doses of purified recombinant PI1a/GNA (A), Ao1b-ProPI1a/GNA (B) or Pro-Hv1a-PI1a/GNA (C). n=20 per treatment.

Figure 12: Ingestion toxicity of recombinant PI1a/GNA, Ao1bPro-PI1a/GNA and Hv1aPro-PI1a/GNA to *Mamestra brassicae*

Percentage survival of 3rd instar *Mamestra brassicae* larvae following ingestion of a single 2 µl droplet containing 20 µg of purified PI1a/GNA (A), Ao1bPro-PI1a/GNA (B) or Hv1aPro-PI1a/GNA (C) fusion proteins. Controls in all cases were sucrose alone (no added protein); 30 µg of either PI1a toxin (mature or modified form) or GNA.

Abbreviations

BB: Binding buffer
ECL: Enhanced chemiluminescence
HRP: Horseradish peroxidase
PBS: Phosphate buffered saline
SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
YPG: Yeast extract peptone glycerol

Materials and Methods

Cloning of recombinant Hv1a, pro-Hv1a and pro-Hv1a/GNA fusion protein

A synthetic gene encoding the mature Hv1a amino acid sequence was assembled using a series of overlapping oligonucleotides, with codon usage optimised for expression in yeast (Fitches *et al.*, 2012). To create an expression construct coding for the mature Hv1a peptide the coding sequence was amplified by PCR using primers with *Pst*I and *Sal*I sites and purified from excised gel slices using a QiaQuick Gel Extraction Kit (Qiagen) as described in the manufacturer's protocol. The extracted DNA fragment was digested (*Pst*I and *Sal*I) and ligated into similarly digested yeast expression vector pGAPZαB (Invitrogen) that had been previously modified to contain a 5' Strep tag in frame with the yeast α-factor pre-pro-sequence. The resulting plasmid was transformed into electrocompetent *E. coli* cells and selected clones were checked for the correct assembly of the construct by gel electrophoresis and DNA sequencing.

The pro-Hv1a coding sequence was amplified by PCR using primers with *Pst*I and *Xba*I sites (Forward: TACTGCAGCAGAAGATACTAGAGCT and Reverse: ATTCTAGAATCACATCTCTTAAC). Gel extracted products were digested with *Pst*I and *Xba*I and ligated into similarly digested yeast expression vector pGAPZαB. The resulting recombinant plasmid was transformed into *E. coli* and selected clones were

checked for correct assembly of the construct by gel electrophoresis and DNA sequencing.

To produce the pro-Hv1a/GNA construct, the pro-Hv1a coding sequence was amplified by PCR using primers with *Pst*I and *Not*I sites (Forward: TACTGCAGCAGAAGATACTAGAGCT and Reverse: ATGCGGCCGC ATCACATCTCTTAAC) and purified by gel electrophoresis as described above. Following restriction by *Pst*I and *Not*I, the PCR product was ligated into a previously generated pGAPZαB plasmid containing the mature GNA coding sequence digested with the same enzymes. Selected clones containing the expression vector encoding the pro-Hv1a/GNA fusion protein were verified by DNA sequencing.

The sequences of the Hv1a constructs are shown below:

Native Hv1a: SPTCIPSGQPCPYNENCCSQSCTFKENENGNTVKRCD

Recombinant Hv1a (alpha factor signal sequence, Hv1a toxin, Strep tag green highlighted region, no pro-region, no carrier):
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKREAEAAWSHPQFEKGLQSPTCIPSGQPCPYNEN
CCSQSCTFKENENGNTVKRCD

Recombinant pro-Hv1a (alpha factor signal sequence, Hv1a toxin, pro-region, no carrier, $(His)_6$ tag):
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKREAEAAAEDTRADLQGGEAAEKVFRRSPTCIPSG
QPCPYNENCCSQSCTFKENENGNTVKRCDALEQKLISEEDLNSAVDHHHHHH

Recombinant Hv1a/GNA (alpha factor signal sequence, Hv1a toxin, no pro-region, carrier):
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKREAEAAASPTCIPSGQPCPYNENCCSQSCTFKEN
ENGNTVKRCDAAADNILYSGETLSTGEFLNYGSFVFIMQEDCNLVLYDVDKPIWATNT
GGLSRSCFLSMQTDGNLVVYNPSNKPIWASNTGGQNGNYVCILQKDRNVVIYGTDR
WATGVD

Recombinant pro-Hv1a/GNA (alpha factor signal sequence, Hv1a toxin, pro-region, carrier, $(His)_6$ tag):

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKREAEAAAEDTRADLQGGEAAEKVFRRSPTCIPSG
QPCPYNENCCSQSCTFKENENGNTVKRCDAAADNILYSGETLSTGEFLNYGSFVFIM
QEDCNLVLYDVDKPIWATNTGGLSRSCFLSMQTDGNLVVYNPSNKPIWASNTGGQN
GNYVCILQKDRNVVIYGTDRWATGVDHHHHHH

Cloning of recombinant Pl1a and Pl1a/GNA fusion proteins

A double stranded DNA incorporating a sequence encoding the mature Pl1a toxin (P83256), with codon usage optimised for yeast, was designed by the inventors and synthesised and supplied by ShineGene Molecular Biotech, Inc. (Shanghai 201109, China; http://www.synthesisgene.com/) in the vector pUC57. Other oligonucleotides were supplied by Sigma Chemical Co.

The Pl1a coding sequence was transferred from pUC57 to the yeast expression vector pGAPZαB (Invitrogen) by digestion with *Pst*I and *Xba*I, isolation of the coding sequence fragment by agarose gel electrophoresis, followed by ligation to pGAPZαB that had been digested with the same enzymes. DNA fragments were purified from excised gel slices using a QiaQuick Gel Extraction Kit (Qiagen). The resulting recombinant plasmid was cloned using standard protocols by transformation of electrocompetent *E. coli* cells. Selected clones were checked for correct assembly of the construct by DNA sequencing. To produce the modified construct for expression of Pl1a two complementary synthetic oligonucleotides encoding the pro-region from U3-agatoxin-Ao1b (Q5Y4V7) were assembled and inserted into the *Pst*I site of the Pl1a expression construct. Correct assembly of the construct (ProAo1b-Pl1a) was checked by DNA sequencing.

To produce a construct encoding the Pl1a/GNA fusion protein, the mature Pl1a coding sequence from a verified expression construct in pGAPZαB was excised by digestion with *Pst*I and *Not*I and purified by agarose gel electrophoresis. A pGAPZαB plasmid containing the fusion protein construct Hv1a/GNA (Fitches *et al.*, 2012) was digested with *Pst*I and *Not*I to remove the Hv1a coding sequence and purified by agarose gel electrophoresis. The Hv1a coding region was subsequently replaced with Pl1a by

ligating the purified fragments and cloning the resulting recombinant plasmid. To produce the modified expression construct for PI1a/GNA containing the pro-region from U3-agatoxin-Ao1b, the PI1a/GNA expression construct was modified as described above; in addition, a pro-region from the pro-Hv1a toxin was also used in a further construct designated as Pro-Hv1a-PI1a. Selected clones containing the expression vector encoding the PI1a/GNA fusion proteins were verified by DNA sequencing. All DNA sequencing was carried out using Applied Biosystems ABI Prism 3730 automated DNA sequencers by DBS Genomics, School of Biological and Biomedical Sciences, Durham University, UK.

The sequences of the PI1a constructs are shown below:

Native PI1a: GCLGEGEKCADWSGPSCCDGFYCSCRSMPYCRCRNNS

Recombinant PI1a (alpha factor signal sequence, PI1a toxin, no pro-region, no carrier, $(His)_6$ tag):
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKREAEAAAGCLGEGEKCADWSGPSCCDGFYCSCR
SMPYCRCRNNSALEQKLISEEDLNSAVDHHHHHH

Recombinant Ao1bPro-PI1a (alpha factor signal sequence, <u>pro-region</u> [Ao1b], PI1a toxin, no carrier, + $(His)_6$ tag):
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKREAEAAA<u>ISYEEGKELFQKER</u>GCLGEGEKCADWS
GPSCCDGYCSCRSMPYCRCRNNSALEQKLISEEDLNSAVDHHHHHH

Recombinant PI1a/GNA (alpha factor signal sequence, PI1a toxin, no pro-region, carrier, $(His)_6$ tag):
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKREAEAAAGCLGEGEKCADWSGPSCCDGFYCSCR
SMPYCRCRNNSAAADNILYSGETLSTGEFLNYGSFVFIMQEDCNLVLYDVDKPIWATN
TGGLSRSCFLSMQTDGNLVVYNPSNKPIWASNTGGQNGNYVCILQKDRNVVIYGTDR
WATGVDHHHHHH

Recombinant Ao1bPro-PI1a/GNA (alpha factor signal sequence, pro-region [Ao1b], PI1a toxin, carrier, $(His)_6$ tag):

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKREAEAAAISYEEGKELFQKERGCLGEGEKCADWS
GPSCCDGCSCRSMPYCRCRNNSAAADNILYSGETLSTGEFLNYGSFVFIMQEDCNLV
LYDVDKPIWATNTGGLSRSCFLSMQTDGNLVVYNPSNKPIWASNTGGQNGNYVCILQ
KDRNVVIYGTDRWATGVDHHHHHH

Recombinant Hv1aPro-PI1a/GNA (alpha factor signal sequence, pro-region [Hv1a], PI1a toxin, carrier, $(His)_6$ tag):

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKREAEAAAEDTRADLQGGEAAEKVFRRGCLGEGE
KCADWSGPCCDGFYCSCRSMPYCRCRNNSAAADNILYSGETLSTGEFLNYGSFVFI
MQEDCNLVLYDVDKPIWATNTGGLSRSCFLSMQTDGNLVVYNPSNKPIWASNTGGQ
NGNYVCILQKDRNVVIYGTDRWATGVDHHHHHH

Expression of Hv1a, pro-Hv1a, Hv1a/GNA and pro-Hv1a/GNA fusion proteins in yeast

pGAPZαB plasmids containing Hv1a, pro-Hv1a, Hv1a/GNA and pro-Hv1a/GNA sequences were amplified in *E. coli*, purified and linearised with *Bln*I (Takara). Linearised plasmids were transformed into *Pichia pastoris* strain SMD1168H (Invitrogen) using the EasyComp Transformation kit (Invitrogen) as described in the manufacturer's protocol. Transformed yeast clones were selected on YPG agar plates (1% yeast extract (w/v), 2% peptone (w/v), 4% glycerol (v/v), 1.5% agar (w/v)) containing zeocin (100 mg/ml). Selected clones were checked for expression by analysis of culture supernatants from small-scale shake flask cultures (10 ml) grown for 2-3 days in YPG–zeocin media at 30°C. Supernatant samples were separated by SDS-polyacrylamide gel electrophoresis; gels were blotted onto nitrocellulose and probed with anti-$(His)_6$ primary antibodies (BioRad) or anti-Strep antibodies, or for Hv1a/GNA and pro-Hv1a/GNA blots were probed with anti-GNA primary antibodies, followed by washing, probing with HRP-conjugated secondary antibodies (BioRad), and detection of bound antibodies by ECL, as described previously (Fitches *et al.*, 2001; 2012).

For protein production selected *P. pastoris* clones containing the integrated Hv1a, pro-Hv1a, Hv1a/GNA and pro-Hv1a/GNA expression cassettes were grown in either a 7.5

L BioFlo 110 bench-top fermenter (New Brunswick Scientific) or a 5 L Bio-Controlly ADI1010 bench-top fermenter (APPLIKON BIOTECHNOLOGY, Holland). YPG cultures (200 ml) of transformed *P. pastoris* were grown for 2–3 days at 30°C with shaking (no zeocin antibiotics) before inoculating 2.5 L of sterile minimal media supplemented with PTM1 salts. Cultivation at 30°C, 30% dissolved oxygen, pH 4.5-5.0 with continuous agitation was carried out with a ramped glycerol feed (5–10 ml/h; total 1.25 l) over a period of 4 days. Culture supernatant was subsequently separated from cells by centrifugation (20 min, 8000 rpm; 4°C), clarified by filtration through 2.7 µM and 0.7 µM glass fibre filters (GFD and GFF; Whatmann). For Hv1a only supernatant was adjusted to 50 mM phosphate buffer containing 0.3 M sodium chloride at pH 8.0 by adding 4x concentrated stock. Recombinant Hv1a was purified on streptactin columns (1 ml) with a flow rate of 0.5 ml/min. Columns were equilibrated in 50 mM phosphate buffer containing 0.3 M sodium chloride at pH 8.0. Strep-tagged Hv1a was eluted from columns using 2.5 mM desthiobiotin (in phosphate buffer; pH 8.0). For all other proteins supernatants were adjusted to 0.02 M sodium phosphate buffer, 0.4 M sodium chloride, pH 7.4 by adding 4x concentrated stock (4X Binding buffer [BB]). Recombinant pro-Hv1a Hv1a/GNA and pro-Hv1a/GNA were purified by nickel affinity chromatography on 5 ml HisTrap crude nickel columns (GE Healthcare) with a flow rate of 2 ml/min. After loading, the columns were washed with 1xBB (50 mM sodium phosphate; 0.4 M sodium chloride) and then with BB containing 0.025 M imidazole, and finally bound recombinant proteins were eluted with BB containing 0.2 M imidazole. In all cases eluted proteins were then checked for purity by SDS-PAGE, dialysed against deionised water using multiple changes to remove all small molecules, and freeze-dried. Concentrations of recombinant proteins were estimated by comparison to known amounts of GNA standards run on SDS-PAGE gels or by BCA analysis using a BCA™ Protein Assay Kit (Thermo Scientific).

Expression of Pl1a and Pl1a/GNA fusion proteins in yeast

pGAPZαB plasmids containing the Pl1a and Pl1a/GNA expression constructs were amplified in *E. coli*, purified and linearised with *Bln*I. Linearised plasmids were transformed into *Pichia pastoris* strain SMD1168H (Invitrogen) using the EasyComp Transformation kit (Invitrogen) as described in the manufacturer's protocol. Transformed yeast clones were plated and selected on YPG agar plates (1% yeast extract (w/v), 2% peptone (w/v), 4% glycerol (v/v), 1.5% agar (w/v)) containing zeocin (100 mg/ml). Selected clones (at least 10 for each construct) were checked for

expression of recombinant proteins by analysis of culture supernatant from small-scale shake flask cultures grown for 2-3 days in YPG–zeocin media at 30°C. Samples of supernatant were separated by SDS-polyacrylamide gel electrophoresis; gels were blotted onto nitrocellulose and probed with anti-$(His)_6$ primary antibodies (BioRad) or anti-GNA primary antibodies, followed by washing, probing with HRP-conjugated secondary antibodies (BioRad), and detection of bound antibodies by ECL.

Selected clones of *P. pastoris* containing the integrated PI1a and PI1a/GNA constructs were grown in a 5 L Bio-Controlly ADI1010 bench-top fermenter (Applikon Biotechnology, Holland). For fermentation, two 100 ml YPG cultures of *P. pastoris* containing toxin or fusion genes were grown for 2–3 days at 30°C with shaking, prior to being used to inoculate 2.5 L of sterile minimal media supplemented with PTM1 salts. Cultivation at 30°C, 30% dissolved oxygen, pH 4.5 -5.0 with continuous agitation was continued with a ramped glycerol feed (5–10 ml/h) over a period of 4 days. Culture supernatant was separated from cells by centrifugation (20 min at 5000 g), and adjusted to 0.02 M sodium phosphate buffer, 0.4 M sodium chloride, pH 7.4 by adding 5x concentrated stock. Recombinant proteins were purified by nickel affinity chromatography on 5 ml HisTrap crude nickel columns (GE Healthcare) with a flow rate of 2 ml/min. After loading, the columns were washed with 0.02 M sodium phosphate buffer, 0.4 M sodium chloride pH 7.4 and the bound proteins were eluted with 0.2 M imidazole in the same buffer. Eluted proteins were checked for purity by SDS-PAGE, dialysed against deionised water using multiple changes to remove all small molecules, and freeze-dried. Concentrations of recombinant proteins were estimated by comparison to known amounts of GNA standards run on SDS-PAGE gels or by BCA analysis using a BCA™ Protein Assay Kit (Thermo Scientific).

Insect bioassays

3rd - 5th instar *Mamestra brassicae* larvae (approximately 30-55 mg in weight) were used for injection bioassays. Larvae were injected with varying doses of Hv1a, pro-Hv1a, pro-Hv1a/GNA, PI1a, Ao1bProPI1a, PI1a/GNA, Ao1bPro-PI1a/GNA, or Hv1aPro-PI1a/GNA (n=20 per dose) in 5µl of PBS (phosphate buffered saline; 0.15 M NaCl, 0.015 M sodium-phosphate buffer, pH 7.2). Larvae for controls were injected with 5 µl 1×PBS. Paralysis and mortality was recorded 12-96 h after injection.

Droplet-feeding assays were carried out to assess the oral activity of Hv1a/GNA, pro-Hv1a/GNA, Pl1a, Ao1bProPl1a, Pl1a/GNA, Ao1bPro-Pl1a/GNA, or Hv1aPro-Pl1a/GNA towards third to fifth instar larvae of *M. brassicae*. Larvae were starved for approximately 24h before feeding in order to encourage droplet consumption. Larvae were fed with a 2 µl droplet containing 20 µg of the above fusion proteins, 30 µg of toxins or 30 µg of GNA, in 1 x PBS solution containing 10% sucrose (w/w). Control larvae were fed on PBS/sucrose droplets containing no added protein. Treated larvae were placed on standard artificial diet after consumption of the droplet.

The insecticidal effects of Hv1a/GNA and pro-Hv1a/GNA to *Acyrthosiphon pisum* (pea aphids) and *Sitobion avenae* (cereal aphids) was assayed by feeding 100 µl liquid artificial diet containing known concentrations of fusion proteins (Prosser and Douglas, 1992), using double parafilm sachets (diet droplet in the middle) to deliver diet to insects. The experiment used 1-2 day-old aphids and survival was assessed daily for six days.

Injection bioassays: *Deroceras reticulatum* (mollusc grey field slug)

Hv1a/GNA and pro-Hv1a/GNA were tested for activity against adult slugs (*Deroceras reticulatum*) by injection into adult slugs (0.2 – 0.3 g). Slugs were chilled at 4°C (for approximately 15 minutes) prior to injection of 25 µg, 50 µg or 100 µg of purified fusion proteins resuspended in 20 µl PBS. Mortality was assessed daily for 7 days.

Statistical analysis

Survival data were analysed using Kaplan–Meier survival analysis, using Prism (v5) software. All other data analysis was carried out using Origin 8.5 graphing and data analysis software. ANOVA analysis (with Bonferroni-Dunn post-hoc tests) was carried out to determine any significant differences between treatments in the parameters measured.

Results

The present inventors have conducted experiments to investigate the effect of inclusion of a pro-region in an expression construct for a toxin on the biological activity of said toxin.

Experiments investigating the toxicity of Hv1a

Introduction

To investigate the effect of inclusion of a pro-region on the toxicity of recombinant toxins, ω-Hexatoxin-Hv1a was used. ω-Hexatoxin-Hv1a is a toxin isolated from the funnel-web spider *Hadroncyhe versuta*. ω-Hexatoxin-Hv1a (or ω-ACTX-Hv1a) is a calcium channel antagonist and it has previously been shown that ω-ACTX-Hv1a can block invertebrate but not vertebrate calcium channels.

Although it has been shown that ω-ACTX-Hv1a can be used on its own as a pesticide when applied topically to caterpillars (Khan *et al.,* 2006), no further evidence for insecticidal activity of the peptide alone has been reported. In patent application PCT/GB2012/000287 the present inventors demonstrated that the toxicity of a recombinant toxin (ω-ACTX-Hv1a) expressed in *Pichia pastoris* could be enhanced by expressing the protein in fusion with the plant lectin GNA, which had previously been shown to cross the gut epithelium and deliver 'passenger' peptides from the gut to the circulatory system of invertebrate animals.

To further investigate how the potency of toxins expressed *in vitro* might be improved, the present inventors analysed the DNA sequences of the genes encoding arthropod toxins. The arthropod toxins utilised in PCT/GB2012/000287 are small, cysteine-rich proteins belonging to several superfamilies of protein sequences (which include toxins from organisms other than arthropods). The encoding genes include two sequences that are not present in the final protein product; a predicted N-terminal signal peptide that is removed during translation and a predicted pro-region, between the signal peptide and the final sequence of the protein as isolated (see Figure 1A).

The present inventors investigated the effect of including the pro-region in the expression construct on the overall toxicity of the recombinant protein.

In the first instance, ω-ACTX-Hv1a was used, as this toxin contains a predicted pro-region in its gene sequence (see Figure 1B).

Synthetic gene constructs, expression and purification of recombinant Hv1a, pro-Hv1a, Hv1a/GNA and pro-Hv1a/GNA

Recombinant Hv1a, pro-Hv1a, Hv1a/GNA and pro-Hv1a/GNA fusion protein constructs were synthesised based on the vector pGAPZαB, which possesses a strong constitutive promoter (*GAPDH*) to direct target gene expression. The pGAPZ vectors are integrating vectors in the expression host *Pichia pastoris* and selected transformants contain the expression construct integrated into the host genome. The constructs for expressing recombinant Hv1a and pro-Hv1a contained a predicted amino acid sequence corresponding to the published sequence for the toxin. For Hv1a, the mature peptide was cloned in frame with the yeast α-factor prepro-sequence and 8 amino acids encoding a Strep tag (i.e. WSHPQFEK linked to the N-terminus of mature Hv1a sequence via a 3 amino acid linker 'GLQ'). For Pro-Hv1a, the N-terminal pro-region was arranged in-frame C-terminal to a sequence encoding the yeast α-factor prepro-sequence, and the construct also contained sequences encoding the myc epitope and $(His)_6$ tag, supplied by the vector, at the C-terminus of the predicted product. For Hv1a/GNA, the mature toxin sequence was fused to the N-terminus of a coding sequence corresponding to residues 1–105 of mature snowdrop lectin (GNA) via a 3 amino acid (AAA) linker peptide. For pro-Hv1a/GNA the synthetic pro-Hv1a coding sequence was fused to the N-terminus of a coding sequence corresponding to residues 1–105 of mature snowdrop lectin (GNA) via a 3 amino acid (AAA) linker peptide. Both fusion protein constructs were also arranged in-frame with the yeast α-factor prepro-sequence, and C-terminal to a sequence encoding $(His)_6$ tag, supplied by the vector. The constructs were assembled by ligation of endonuclease digested DNA and were checked by DNA sequencing after cloning.

Sequence verified clones, containing recombinant Hv1a, pro-Hv1a, Hv1a/GNA or pro-Hv1a/GNA were transformed into the *P. pastoris* protease-deficient strain SMD1168H and selected using the antibiotic zeocin. Selected clones were grown in shake flask cultures for 3-4 days at 30℃ and culture supernatants were analysed for the expression of recombinant proteins using western blotting. This enabled highly-expressing clones to be selected for production by bench-top fermentation. The majority of the analysed transformed yeast clones showed evidence of protein expression as judged by the presence of immunoreactive bands with the expected size on western blots (results not shown).

Fermentation of the selected clones was carried out in bioreactors under controlled environmental conditions. The use of the pGAP alpha factor secretory signal that

directs the secretion of expressed proteins out of the cells and into the growth media, enabled subsequent purification of recombinant proteins from fermented culture supernatants. Supernatants were obtained by centrifugation, clarified by filtration and recombinant proteins were subsequently purified by affinity chromatography (Streptactin for Hv1a and nickel affinity for pro-Hv1a and pro-Hv1a/GNA). Eluted peaks containing target proteins were desalted by dialysis and lyophilised. For yields of recombinant proteins, Hv1a was produced at approximately 5-10 mg/L culture supernatant; pro-Hv1a was produced at approximately 40 mg/L, as estimated by BCA quantification and Hv1a/GNA at approximately 40 mg/L and pro-Hv1a/GNA at approximately 21 mg/L, as estimated by semi-quantitative SDS-PAGE.

As shown in Figure 2A (lanes 3 and 4), 5' Strep tagged mature purified Hv1a separated on SDS-PAGE gels as a single protein of approximately 6.5 kDa, comparable to the predicted molecular mass of 5.47 kDa (gels were not optimised for the separation of low molecular weight proteins). Purified recombinant pro-Hv1a was separated using Tris-Tricine SDS-PAGE gels and analysed by both staining for total protein and western blotting using anti-His antibodies (Figure 2B & C). The recombinant toxin pro-Hv1a gave a major protein band at approximately 4 kDa and further weaker bands in molecular mass range 6-16 kDa on Tris-Tricine gels. The dominant band of approximately 4 kDa is not immunoreactive with anti-$(His)_6$ antibodies (Figure 2C) and the molecular mass is consistent with the predicted mass (4.06 kDa) following cleavage of the C-terminal tag region. Pull down of non-His tagged proteins along with His-tagged proteins from nickel affinity columns has previously been observed for other recombinant proteins recovered from *Pichia* supernatants. The 10 kDa protein that shows positive immunoreactivity with anti-$(His)_6$ antibodies corresponds to the predicted mass (9 kDa) for recombinant Hv1a including the pro-region, suggesting that cleavage of the pro-region is incomplete during processing by yeast cells. The 14 kDa band also immunoreactive with anti-$(His)_6$ antibodies may represent a dimeric form of Hv1a given that the predicted mass of Hv1a containing the C-terminal His region but no pro-region is 6.74 kDa.

Lyophilised samples of purified pro-Hv1a/GNA, Hv1a/GNA and MODHv1a/GNA were analysed on SDS-PAGE gels (Figure 3). Two bands of approximately 19 kDa and 14 kDa were observed for all fusion proteins. The predicted molecular mass for Hv1a/GNA is 16.27 kDa, and for pro-Hv1a/GNA without the pro-region but containing a

$(His)_6$ tag is 16.95, both slightly less than the observed 19 kDa band. However, the identical separation of pro-Hv1a/GNA and Hv1a/GNA on SDS-PAGE gels suggests that the pro-region has been cleaved from pro-Hv1a/GNA during processing by *P. pastoris* cells. The predicted mass for intact MODHv1a/GNA is 17.09 kDa and correspondingly this protein runs as a slightly larger protein as compared to Hv1a/GNA and pro-Hv1a/GNA due to the presence of an additional histidine tag that is not present in Hv1a/GNA. In all cases the smaller 14 kDa band is immunoreactive with GNA antibodies (results not shown) and corresponds in size to GNA from which the Hv1a toxin has been cleaved. As observed previously for Hv1a/GNA (Fitches *et al.,* 2012), the ratio of intact pro-Hv1a/GNA fusion protein to cleaved GNA was estimated as approx. 1:1 as judged by Coomassie blue staining on SDS-PAGE gels, whereas modification of the Hv1a sequence in MODHv1a/GNA results in a higher recovery of intact fusion protein (ratio intact:cleaved 2:1). Quantification of the Hv1a/GNA fusion proteins was based on comparative band intensity with GNA standards of known concentration as shown in Figure 3.

Injection toxicity of recombinant Hv1a, pro-Hv1a, Hv1a/GNA and pro-Hv1a/GNA fusion protein to cabbage moth* (Mamestra brassicae) *larvae

Injections of Hv1a at doses of up to 100 µg of the recombinant toxin did not result in any larval mortality with survival comparable to controls (n=40; survival >90%). This demonstrated that the expression of mature Hv1a peptide without an N-terminal pro-region resulted in the production of biologically inactive toxin, suggesting that the toxin was incorrectly processed and/or folded during synthesis by yeast cells. By contrast, injections of newly eclosed 3rd-4th (~30-40 mg) and 5th instar (~45-55 mg) *M. brassicae* larvae with either pro-Hv1a, Hv1a/GNA, MODHv1a/GNA or pro-Hv1a/GNA led to significant larval mortality.

As shown in Figure 4A the effects of recombinant pro-Hv1a after injection into 5th instar larvae were dose dependent. Injection doses of 10 µg and 20 µg pro-Hv1a/insect resulted in complete mortality 24 hours post injection and injection of 5 µg/insect resulted in 80% mortality 24 hours after injection. At the lowest dose of 1.25 µg/insect, all insects displayed flaccid paralysis and a temporary absence of feeding although some paralysed insects recovered and were able to resume feeding 2-3 hours after injection. From these assays, the estimated LD_{50} (lethal dose; 48 hours) for the

recombinant pro-Hv1a was 25 µg/g insect. As summarised in Table 1 this is approximately 3–fold lower than that previously published for recombinant Hv1a toxin produced in *E. coli*, whose LD_{50} (72 hours) was ~69 µg/g insect.

As shown in Figure 4B & C injections of 3-4th and 5th instar *M. brassicae* larvae with pro-Hv1a/GNA demonstrated increased toxicity as compared to Hv1a/GNA. For example injections of 10 µg Hv1a/GNA into 5th instar larvae resulted in 50% mortality after 72 hours as compared to 100% mortality recorded 24 hours after injection of the same dose of pro-Hv1a/GNA. Significant larval mortality (75%) was observed at a pro-Hv1a/GNA dose of 2.5 µg/insect whereas injections of 5 or 10 µg Hv1a/GNA did not result in any significant levels of mortality for 5th instar larvae. Similar results were observed following the injection of smaller 3-4th instar larvae (Figure 4B). As shown in Table 1 LD_{50} values estimated for pro-Hv1a/GNA were some 10-fold lower (4.6 µg/g insect) as compared to an LD_{50} of 55 µg/g insect for Hv1a/GNA. This demonstrates that the addition of the pro-region to the Hv1a/GNA construct results in the production of fusion protein that is significantly more toxic as compared to protein derived from a construct encoding the mature toxin sequence fused to GNA. More surprisingly, the LD_{50} value of 4.6 µg/g insect calculated for pro-Hv1a/GNA is some 5-fold lower than 25 µg/g insect estimated for pro-Hv1a. This shows that linkage of the pro-Hv1a to GNA results in a protein with higher biological activity than either pro-Hv1a alone, or Hv1a/GNA without the pro-region. The LD_{50} value of 4.6 µg/g insect calculated for pro-Hv1a/GNA is also over 2-fold lower than the literature value for native Hv1a (as opposed to recombinant Hv1a; see table 1). This literature value represents the LD_{50} value in *Heliothis*, a species of the same insect order as *M. brassicae*. We hypothesise that fusing GNA to pro-Hv1a further acts to facilitate correct processing and folding of the Hv1a toxin by *P. pastoris* cells, allowing for further increases in biological activity to be attained. Injections of GNA alone at up to 40 µg/insect do not result in mortality of *M. brassicae* larvae.

Table 1: Toxicity of recombinant toxins and fusion proteins in injection bioassays with *Mamestra brassicae* larvae

	Hv1a (literature)	Hv1a	Pro-Hv1a	Hv1a/GNA	Pro-Hv1a/GNA	Hv1a (*E. coli*)
LD_{50}	12 µg/g (*Heliothis sp.)**	>1000 µg/g	25 µg/g (48h) < 25 µg/g (72h)	55 µg/g (72h)	4.6 µg/g (48h)	69 µg/g (72h)

* Data not available for *M. brassicae*

Ingestion toxicity of recombinant Hv1a, pro-Hv1a and pro-Hv1a/GNA fusion protein to cabbage moth (*M. brassicae*) larvae

The oral activity of proHv1a/GNA and Hv1a/GNA was assessed by feeding 2 µl droplets containing 20 µg of fusion protein to newly eclosed third instar *M. brassicae* larvae. Control treatments were 20 µg of either GNA or pro-Hv1a, in addition to a no-added protein control group. As shown in Figure 5 and Table 2, significant effects were observed only for larvae fed on pro-Hv1a/GNA, with 90% mortality recorded 5 days after the ingestion of a single droplet of fusion protein. By contrast, mortality was only 30% for the Hv1a/GNA fusion protein, only slightly greater than the 20% and 15% mortality observed for GNA and pro-Hv1a treatments, respectively. Similar results were observed in assays where a single dose of 20 µg of pro-Hv1a/GNA fusion protein was found to cause 30% mortality of fifth instar larvae over 4 days, whereas no mortality was observed for larvae fed on either 20 µg of Hv1a/GNA, or pro-Hv1a (results not shown).

Table 2: Toxicity of recombinant toxins and fusion proteins in oral feeding assays with *Mamestra brassicae* larvae

	Pro-Hv1a	Hv1a/GNA	Pro-Hv1a/GNA	Hv1a (*E. coli*)
Percentage Survival	85% (5d) 600 µg/g	70% (5d) 500 µg/g	10% (5d) 500 µg/g	100% (5d) 180 µg/g

Ingestion toxicity of recombinant Hv1a, pro-Hv1a and pro-Hv1a/GNA fusion protein to pea (A. pisum) and cereal (Sitobion avenae) aphids

Recombinant pro-Hv1a protein, pro-Hv1a/GNA and Hv1a/GNA were tested for oral activity against pea and cereal aphids by incorporation into artificial diet at concentrations of 0.125 mg – 0.75 mg/ml (125 - 750 ppm). As observed for lepidopteran larvae, purified pro-Hv1a/GNA was found to be significantly more toxic than Hv1a/GNA to both aphid species (Figure 6A & B). Pro-Hv1a/GNA at 750 ppm caused 100% mortality of pea aphids after 3 days, whereas the same dose of Hv1a/GNA resulted in only 50% mortality after 8 days of feeding. At a lower dose of 500 ppm, mortality after 8 days of feeding was 100% for pea aphids fed on pro-Hv1a/GNA as compared to 20% for Hv1a/GNA.

Pro-Hv1a/GNA was also found to be significantly more toxic than Hv1a/GNA to cereal aphids. As shown in Figure 6B, 100% mortality was recorded for cereal aphids fed on diets containing 250 ppm of pro-Hv1a/GNA for 7 days as compared to 60% for Hv1a/GNA fed aphids. Cereal aphids appear to be more susceptible to pro-Hv1a/GNA than pea aphids as significant levels of mortality were observed at levels as low as 125 ppm pro-Hv1a/GNA (80% mortality after 2 days of feeding) whereas no mortality was recorded for pea aphids fed on the same dose of fusion protein.

***Injection toxicity of recombinant MODHv1a and Pro-Hv1a/GNA fusion protein to grey field slugs* (Deroceras reticulatum)**

MODHv1a/GNA and Pro-Hv1a/GNA were tested for activity against slugs (*D. reticulatum*) by injection into adult slugs (~0.2 g). MODHv1a/GNA corresponds to the modified form of Hv1a/GNA, where a single amino acid change at the C-terminus of Hv1a has been shown to improve expression of intact fusion protein but has equivalent toxicity to Hv1a/GNA. Slugs were chilled at 4°C (for ~15 minutes) prior to injection of 25, 50 or 100 µg of purified Hv1a/GNA resuspended in 20 µl PBS. Mortality was assessed daily for 7 days. Figure 7 shows dose dependent mortality observed for both treatments. Mortality was significantly greater for pro-Hv1a/GNA as compared to MODHv1a/GNA for all doses injected ($P<0.05$; Mantel Cox tests). For example, 100% mortality was recorded 3 days after injection of 50 µg of pro-Hv1a/GNA as compared to 10% mortality observed 5 days after injection of 50 µg of MODHv1a/GNA.

Experiments investigating the toxicity of PI1a

Introduction

Results obtained for the Hva1/GNA fusion protein were extended by taking a toxin protein whose gene sequence did not include a predicted pro-region and incorporating a pro-region into the expression construct based on similar sequences in the global protein database. The toxin δ-amaurobitoxin-PI1a from the spider *Pireneitega luctuosa* was utilised.

Expression and purification of recombinant PI1a and PI1a/GNA

Expression constructs for the production of recombinant proteins in *Pichia pastoris* were based on the vector pGAPZαB, which contains a strong constitutive promoter used to direct expression of the recombinant protein and which is designed to integrate into the host genome at the *GAPDH* locus, giving stable transformants. Expression constructs for the production of recombinant PI1a contained a synthetic coding sequence corresponding to the published amino acid sequence for the toxin designated PI1a, arranged in-frame C-terminal to a sequence encoding the yeast α-factor prepro- sequence. Constructs containing toxin pro-regions had these inserted between the yeast α-factor prepro- sequence and the PI1a toxin sequence. The pro-regions used were taken from the closely related toxin U3-agatoxin-Ao1b from the spider *Agelena orientalis* (a cDNA sequence including the pro-region is not available for PI1a), designated Ao1bPro-PI1a, and from the pro-region for the Hv1a atracotoxin, as previously described, designated Hv1aPro-PI1a. The expression constructs also contained C-terminal sequences encoding the myc epitope and $(His)_6$ tag, supplied by the vector.

Three expression constructs were created for the production of recombinant PI1a/GNA fusion protein and all contained the mature PI1a coding sequence fused to the N-terminus of a coding sequence corresponding to residues 1–105 of mature snowdrop lectin (GNA) via a 3 amino acid linker peptide; again, the fusion proteins were arranged in-frame C-terminal to the α-factor prepro- sequence, and N-terminal to a sequence encoding the $(His)_6$ tag, supplied by the vector. Modified fusion protein constructs also contained the pro-regions of Ao1b and Hv1a as described above, inserted between the yeast α-factor prepro- sequence and the mature coding sequence of PI1a; they were

designated Ao1bPro-PI1a/GNA and Hv1aPro-PI1a/GNA. The constructs were assembled by restriction-ligation and were checked by DNA sequencing after cloning.

Verified clones of expression constructs were transformed into the protease-deficient *P. pastoris* strain SMD1168H, using antibiotic (zeocin) selection for transformants. Approximately 50 resistant colonies were obtained for each expression construct. Culture supernatant from selected clones grown in shake-flask cultures was analysed for production of recombinant proteins by western blotting, to allow selection of clones producing the highest levels of PI1a and PI1a/GNA fusion proteins. Screening of large numbers of transformed yeast clones was not necessary, since most clones were expressing recombinant proteins, as judged by the presence of immunoreactive bands of the expected size on western blots of culture supernatants (results not shown).

For each construct, the best-expressing clone of those screened in small-scale cultures was selected for large-scale protein production by bench top fermentation. Culture supernatants were purified by nickel affinity chromatography and eluted peaks were desalted by dialysis and lyophilized. Yields of recombinant proteins were comparable to other fusion proteins prior to optimisation; PI1a was produced at approximately 26 mg/L, Ao1bPro-PI1a at approximately 32 mg/L, PI1a/GNA at approximately 21 mg/L, Ao1bPro-PI1a/GNA at approximately 32 mg/L and Hv1aPro-PI1a at approximately 13 mg/L as estimated by semi-quantitative analysis.

Purified recombinant PI1a toxins were analysed by SDS-PAGE and western blotting (Figure 8). The recombinant toxin PI1a produced by the construct without the added pro-region ran as a closely spaced double band at an indicated molecular weight of approximately 18 kDa on SDS-PAGE gels (Figure 8A); both bands were immunoreactive with anti-$(His)_6$ antibodies. The predicted molecular weight of recombinant PI1a, including the tag sequences is 6.87 kDa. The double band of toxin was reproducible with different gels, samples and use of reducing agents prior to electrophoresis, but was considered to be an artefact of the gel system, possibly as a result of poor binding of SDS to the polypeptide. When the same samples were pre-treated with 6 M urea, PI1a gave a single band at an indicated molecular weight of 14 kDa (Figure 8B); the shift in mobility is indicative of gel artefacts, and the single band indicates homogeneity of the product. Further analysis on urea-containing gels gave single bands for PI1a, with indicated molecular weights of ~11 kDa without blocking

cysteine residues and ~9 kDa after treatment with iodoacetamide to block cysteine residues; these results are diagnostic of incorrect molecular weights under "normal" conditions due to residual secondary structure and interactions between cysteine residues prior to or during electrophoresis. N-terminal sequencing verified incomplete processing of the Kex2 pGAPZαB cleavage site resulting in an additional glutamic acid and alanine residue at the N-terminus and a predicted product mass of 7.07 kDa. Interestingly, PI1a produced by the modified construct incorporating the pro-region (Ao1bPro-PI1a) ran as a closely spaced double band at ~9 kDa under "normal" SDS-PAGE conditions, with some evidence of a diffuse band at higher molecular weight (Figure 8C). The predicted molecular mass of the peptide including the additional pro-region is 8.6 kDa. N-terminal sequencing confirmed that the pro-region was present in the protein product and that cleavage had occurred between alanine and the primary residue of the Ao1b pro-region isoleucine giving a predicted molecular mass of 8.46 kDa.

The “normal” PI1a/GNA fusion protein (i.e. derived from a construct that did not contain an additional pro-region) separated on SDS-PAGE gels as two major proteins of indicated sizes of 18 and 21 kDa (Figure 9A). The 18 kDa protein, immunoreactive with anti-GNA antibodies (results not shown) corresponded in mass to that predicted for recombinant PI1a/GNA (17.1 kDa). The 21 kDa protein was also immunoreactive with anti-GNA antibodies, and had an identical N-terminal sequence to the 18 kDa band. Treatment with the deglycosylating enzyme PNGase F, which cleaves carbohydrate side chains attached to Asn residues through N-glycosidic bonds, removed this band, while the intensity of the "correct" band for the PI1a/GNA fusion protein increased as a result of the treatment (Figure 9B). This result suggests that the extra band is due to "core" glycosylation of the fusion protein by *P. pastoris* during synthesis and secretion. GNA contains no potential N-glycosylation sites, but the PI1a toxin sequence contains a potential N-glycosylation site (N-X-S/T) at Asn-35. The N-terminal sequence of the single band was determined as E-A-A-A-G-, as expected for the fusion protein after removal of the yeast α-factor prepro-region during translation and secretion from *P. pastoris*. In addition a small amount of a band at an indicated molecular weight similar to recombinant GNA (12.7 kDa), which was immunoreactive to anti-GNA antibodies (results not shown), suggesting a small amount of cleavage of the fusion protein into its components was occurring during production and purification. The ratio of intact

PI1a/GNA fusion protein to cleaved GNA was estimated as ~30:1 as judged by Coomassie blue staining on SDS-PAGE gels.

Both of the PI1a/GNA fusion proteins derived from constructs containing additional pro-region sequences (i.e. Ao1bPro-PI1a/GNA and Hv1aPro-PI1a/GNA) separated on SDS-PAGE gels as two major staining bands of approximately 17 and 21 kDa (Figure 9C & D). The smaller 17 kDa protein corresponds in mass to that predicted for Ao1bPro-PI1a/GNA and Hv1aPro-PI1a/GNA (16.94 kDa) following removal of the pro-region, suggesting that in both cases the pro-region is removed during processing by yeast cells. The larger 21 kDa protein band is most likely to represent glycosylated protein, as was observed for PI1a/GNA. Ao1bPro-PI1a/GNA and Hv1aPro-PI1a/GNA both expressed as 100% intact fusion protein with no evidence by SDS-PAGE and western blotting for cleavage between the toxin and GNA sequences.

Injection toxicity of recombinant PI1a and Ao1bPro-PI1a protein to cabbage moth* (M. brassicae) *larvae

Newly eclosed 5th instar larvae (~45-55 mg in weight) of *M. brassicae* were injected with purified recombinant proteins to assess and compare *in vivo* activity of the toxins and fusion proteins. Figure 10A shows survival of larvae following injection with PI1a and Figure 10B shows survival following injection of comparable doses of PI1a and Ao1bPro-PI1a. Larvae injected with PI1a toxin all displayed flaccid paralysis within 1 – 2 hours (little mobility and almost a complete absence of feeding). Most mortality was observed within the first 24 hours. After a period of paralysis, some insects showed progressive recovery and were able to recommence feeding. The effects of PI1a were dose dependent, with mortality after 24 hours ranging from 75% at 20 µg toxin/insect to 20% at 1.25 µg toxin/insect. Even at high doses of toxin, complete mortality after 72 hours was not observed. From these assays, the estimated LD_{50} (48 hours) for the recombinant PI1a was 4.1 µg/insect or 82 µg/g insect, based on an average larval weight of 50 mg (see Table 3). The literature LD_{50} value for native PI1a (as opposed to recombinant PI1a) is 9.5 µg/g. This literature value represents the LD_{50} value in *Spodoptera*, a species of the same insect order as *M. brassicae*.

Recombinant PI1a produced from the modified expression construct, including the pro-region from U3-agatoxin-Ao1b, showed similar toxic effects to PI1a, but was

consistently more effective at lower doses than PI1a produced from the construct without this additional sequence (Figure 10B). Again, the major effects of Ao1bPro-PI1a on mortality were observed during the first 24 hours, with mortality ranging from 80% at 10 µg toxin/insect to 30% at 1.25 µg toxin/insect. In these assays, there was a trend for mortality caused by toxin produced by the modified construct to continue to increase to 72 hours and the highest dose of toxin (10 µg toxin/insect) caused 100% mortality at 72 hours. Assays carried out at the same time with PI1a produced by the unmodified construct gave similar results to the previous assay and direct comparison between the two samples in the same assay showed that differences between PI1a produced by the unmodified and modified constructs were statistically significant when identical dose survival curves were analysed (Figure 10B). The estimated LD_{50} (48 hours) for recombinant PI1a produced from the modified construct (Ao1bPro-PI1a) was ~1.0 µg/insect, or 21 µg/g insect based on a mean larval weight of 50 mg; this is equivalent to an increase in toxicity of ~4-fold (see Table 3).

Table 3: Toxicity of recombinant toxins and fusion proteins in injection bioassays with *Mamestra brassicae* larvae

	PI1a (literature)	**PI1a**	**Ao1bPro-PI1a**	**PI1a/GNA**	**Ao1bPro-PI1a/GNA**	**Hv1aPro-PI1a/GNA**
LD_{50}	9.5 µg/g (*Spodoptera sp.*)*	82 µg/g	21 µg/g	11 µg/g (28 µg/g fusion)	7.6 µg/g (19 µg/g fusion)	<5 µg/g (<12 µg/g fusion)

* data not available for *M. brassicae*

The PI1a/GNA fusion protein also caused paralysis and mortality when injected into *M. brassicae* larvae and was significantly more effective than toxin alone (Figure 11A). When larvae were injected with 1.25-10 µg fusion protein/insect (equivalent to 0.50-4.0 µg PI1a/insect, since the molecular weight of recombinant PI1a is ~0.404 of that of the PI1a/GNA fusion protein), significant mortality was observed at all doses, and complete mortality after 24 hours was observed at the highest dose (Figure 11A). As with PI1a, most mortality was observed within the first 24 hours of the assay and effects were dose dependent, ranging from 100% mortality at 10 µg fusion protein/insect to 35% mortality at 1.25 µg fusion protein/insect. Mortality at this lowest dose of fusion protein increased to 65% after 72h whereas mortality from injection of 1.25 µg toxin alone did not change from 20% between 24 and 72 hours. From these assays, the estimated LD_{50} (48 hours) for the recombinant PI1a/GNA fusion protein was 1.4 µg/insect, or 28

µg/g insect, based on a mean larval weight of 50 mg. The LD_{50} is equivalent to 0.56 µg PI1a toxin per insect, making the PI1a/GNA fusion protein ~7 times as active as the toxin produced by the unmodified construct, and ~2 times as active as the toxin produced by the modified Ao1b-Pro-PI1a construct, on a molar basis. A similar ratio is obtained by using mortality figures at 72 hours. Direct comparisons of mortality produced by identical doses of toxin and fusion proteins show that the three treatments are different from each other, and from control, at $p<0.0001$ (ANOVA). In all these assays, no mortality of control-injected insects was observed over 72 hours.

As observed with PI1a toxin, addition of the Ao1b pro-region to the PI1a/GNA fusion protein expression construct resulted in a protein product with enhanced biological activity (Figure 11B). The fusion protein product derived from this construct had an estimated LD_{50} (48 hours) of 0.94 µg/insect, with increased mortalities at all doses except the highest. Addition of an alternative pro-region from the pro-HV1a toxin, to the PI1a/GNA expression construct also enhanced the biological activity of the resulting fusion protein (Figure 11C); this protein had an estimated LD_{50} (48 hours) of <0.6 µg/insect, although overall mortality values were similar to the pro-Ao1b-PI1a/GNA fusion protein. These data suggest that a two-fold increase in toxicity can be obtained by including pro-regions in the expression constructs for PI1a / GNA.

Ingestion toxicity of recombinant PI1a/GNA, AolbPro-PI1a/GNA and Pro-Hv1aPI1a/GNA proteins to cabbage moth* (M. brassicae) *larvae

A similar increase in toxicity of fusion proteins derived from expression constructs including pro-regions to that observed in injection assays was also observed in droplet feeding assays with 3rd stadium *M. brassicae* larvae (Figure 12). Following ingestion of a single 2 µl droplet containing 20 µg of fusion protein, mortality after 5 days was 40% for PI1a/GNA; 50% for AolbPro-PI1a/GNA and 70% for Hv1aPro-PI1a/GNA (data summarised in Table 4). Minimal reductions in survival (0-20%) were observed for control treatments where larvae were fed on 30 µg toxin or GNA and survival curves for controls were significantly different to fusion protein treatments. This provides further evidence that the addition of pro-regions to the PI1a/GNA construct results in increased biological activity. As for injection studies the use of the Hv1a pro-region was seen to result in the greatest enhancement of toxicity over the non-modified PI1a/GNA fusion protein.

Table 4: Toxicity of recombinant toxins and fusion proteins in oral feeding assays with *Mamestra brassicae* larvae

	Pl1a (recombinant)	Ao1bPro-Hv1a	Pl1a/GNA	Ao1bPro-Pl1a/GNA	Hv1aPro-Pl1a/GNA
Percentage Survival	90% (5d) 400 µg/g	80% (5d) 400 µg/g	60% (5d) 500 µg/g	50% (5d) 500 µg/g	30% (5d) 500 µg/g

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CLAIMS:

1. A method of increasing the biological activity of a recombinant toxin, the method comprising:

providing a nucleic acid construct comprising:

(i) a toxin sequence, or a fragment or variant thereof, linked to

(ii) a pro-region, or fragment or variant thereof; and

optionally expressing the recombinant toxin.

2. A nucleic acid construct comprising:

(i) a pro-region, or fragment or variant thereof; and

(ii) a site adjacent to the pro-region into which a toxin gene sequence, or fragment or variant thereof may be inserted.

3. The nucleic acid construct according to claim 2 further comprising the toxin gene sequence or fragment or variant thereof inserted into the site adjacent to the pro-region.

4. A host cell comprising the nucleic acid construct according to claim 3.

5. The host cell according to claim 4 wherein the cell is the yeast *Pichia pastoris*.

6. A method of producing a recombinant toxin with increased biological activity, the method comprising

culturing a host cell according to claim 4 or 5 under conditions suitable for expression of the recombinant toxin.

7. The methods, nucleic acid construct or host cell according to any of claims 1 or 3-6 wherein the toxin is a pesticide toxin.

8. The methods, nucleic acid construct or host cell according to any of claims 1 or 3-7 wherein the toxin is derived from arthropods, molluscs or other invertebrates.

9. The methods, nucleic acid construct or host cell according to any of claims 1 or 3-8 wherein the toxin is an arthropod toxin, optionally, ω-ACTX-Hv1a or κ-ACTX-Hv1c

from *Hadronyche versuta*, δ-amaurobitoxin-PI1a from *Pireneitega luctuosus*, *Segestria florentina* toxins SfI1-8, *Buthus mesotamulus* toxin ButaIT, theraphotoxin Ec2a from *Eucratoscelus constrictus*, cyrtoautoxin As1a from *Apomastus schlingeri* or sicaritoxin Li1a from *Loxosceles intermedia*.

10. The methods, nucleic acid construct or host cell according to any of claims 1 or 3-9 wherein the toxin comprises a peptide of 20-100 amino acid residues.

11. The methods, nucleic acid construct or host cell according to any of claims 1 or 3-10 wherein the toxin is ω-ACTX-Hv1a, or a fragment or variant thereof.

12. The methods, nucleic acid construct or host cell according to any of claims 1 or 3-10 wherein the toxin is δ-amaurobitoxin-PI1a, or a fragment or variant thereof.

13. The methods, nucleic acid construct or host cell according to any preceding claim wherein the pro-region is a sequence between a signal peptide sequence and a mature protein N-terminus, optionally wherein the pro-region is identifiable by the steps of:

comparing the sequence determined for a protein isolated from its normal source with the sequence predicted by the gene encoding it;

identifying N-terminal sequences not present in the final protein product;

identifying a signal peptide sequence in the N-terminal sequence not present in the final protein product; and

identifying a pro-region sequence between the signal peptide sequence and the mature protein N-terminus, optionally by removal of the signal peptide sequence from the N-terminal sequence not present in the final protein product.

14. The methods, nucleic acid construct or host cell according to any preceding claim wherein the pro-region is rich is acidic amino acid residues.

15. The methods, nucleic acid construct or host cell according to any of claims 1, 3-11, 13 or 14 wherein the pro-region is associated with the toxin in its naturally occurring sequence.

16. The methods, nucleic acid construct or host cell according to any of claims 1 or 3-14 wherein the pro-region is not associated with the toxin in its naturally occurring sequence.

17. The methods, nucleic acid construct or host cell according to any preceding claim wherein the pro-region comprises the amino acid sequence EDTRADLQGGEAAEKVFRR, or a fragment or variant thereof.

18. The methods, nucleic acid construct or host cell according to any of claims 1 to 16 wherein the pro-region comprises the amino acid sequence ISYEEGKELFQKER, or a fragment or variant thereof.

19. The methods, nucleic acid construct or host cell according to any preceding claim wherein the nucleic acid construct further comprises the sequence of a carrier protein, or a fragment or variant thereof.

20. The methods, nucleic acid construct or host cell according to claim 19 when dependent on any of claims 1 or 3-18 wherein the sequence of the carrier protein is fused to the toxin protein sequence, thereby generating a fusion protein, when expressed.

21. The methods, nucleic acid construct or host cell according to claim 19 or 20 wherein the carrier protein is a lectin.

22. The methods, nucleic acid construct or host cell according to any of claims 19-21 wherein the carrier protein is a plant lectin selected from: snowdrop lectin (GNA), garlic lectin *Allium sativum*, pea lectin *Pisum sativum* (P-lec), peanut lectin *Arachis hypogaea*, french bean lectin (PHA, *Phytohaemagglutinin*), or a fragment or variant thereof.

23. The methods, nucleic acid construct or host cell according to any of claims 19-22 wherein the carrier protein is GNA.

24. A nucleic acid construct comprising:

(i) a pro-region, or fragment or variant thereof; and

(ii) a sequence of a carrier protein, or a fragment or variant thereof.

25. The methods, nucleic acid constructs or host cell according to any preceding claim wherein the nucleic acid constructs are expression constructs.

26. The methods, nucleic acid constructs or host cell according to any preceding claim wherein the nucleic acid constructs further comprise a sequence which encodes an affinity tag.

27. The methods, nucleic acid constructs or host cell according to claim 26 wherein the affinity tag is a His-tag.

28. A pesticide composition comprising a toxin protein produced according to one of the methods described in claims 1, 6-23 or 25-27.

29. The pesticide composition according to claim 28, wherein the composition is in the form of a solution, emulsion, spray, suspension, powder, foam, paste, granule, aerosol, capsule or other finely or coarsely divided material or impregnant for natural or synthetic material.

30. The pesticide composition according to claim 28 or 29 wherein the composition is in the form of a spray or suspension in admixture with suitable diluents, adjuvants, preservatives, dispersants, solvents and/or emulsifying agents.

31. The pesticide composition according to any of claims 28-30 wherein the composition is in intimate or physical admixture together with other known insecticides, growth promoting or regulating substances, herbicides, fungicides and/or synergistic agents.

32. The pesticide composition according to any of claims 28-31 wherein the composition comprises a toxin protein in an amount of between 0.001% and 99% by weight preferably between 0.5% and 98% by weight, more preferably between 1.0% and 95% by weight.

33. A process for the preparation of a pesticide composition according to any of claims 28-32 which comprises the admixture of an amount of toxin protein produced according to one of the methods described in claims 1, 6-23 or 25-27 with one or more suitable carriers, diluents, adjuvants, preservatives, dispersants, solvents, emulsifying agents in effective pesticidal amount.

34. A method of preventing or treating a pest infection of a plant comprising applying a quantity of toxin protein produced according to one of the methods described in claims 1, 6-23 or 25-27 or a pesticide composition according to any of claims 28-32 to the plant or its locus of growth; or introducing to the plant the nucleic acid construct according to any of claim 3, or claims 7-23 or 25-27 when dependent on claim 3.

35. The method according to claim 34 wherein the pest is a mollusc pest, optionally a slug or snail, optionally a grey field slug.

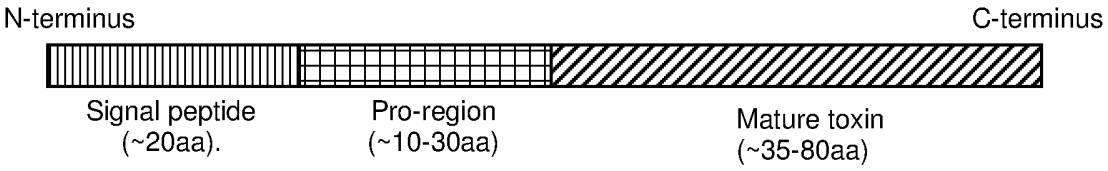
36. The molluscicide bait composition comprising a toxin protein produced according to one of the methods described in claims 1, 6-23 or 25-27 and/or a pesticide composition according to any of claims 28-32.

37. A transgenic plant or progeny thereof comprising a nucleic acid construct as described in any one of claim 3, or claims 7-23 or 25-27 when dependent on claim 3 capable of expressing a toxin with increased biological activity.

38. Use of a nucleic acid construct according to any of claim 3, or claims 7-23 or 25-27 when dependent on claim 3 or a toxin protein produced by one of the methods described in claims 1, 6-23 or 25-27 in the manufacture of a pesticide or a transgenic plant cell or plant.

39. Use of a pesticide according to any of claims 28-32 to destroy or debilitate one or more pests.

A



B

EDTRADLQGGEAAEKVFRRSPTCIPSGQPCPYNENCCSQSCTFKENENGNTVKRCD

Figure 1: Schematic of gene structure of toxins containing pro-regions
(A) Schematic gene structure of toxin proteins containing pro-regions. **(B)** Sequence of spider toxin, Hv1a. Boxed amino acid sequence corresponds to pro-region.

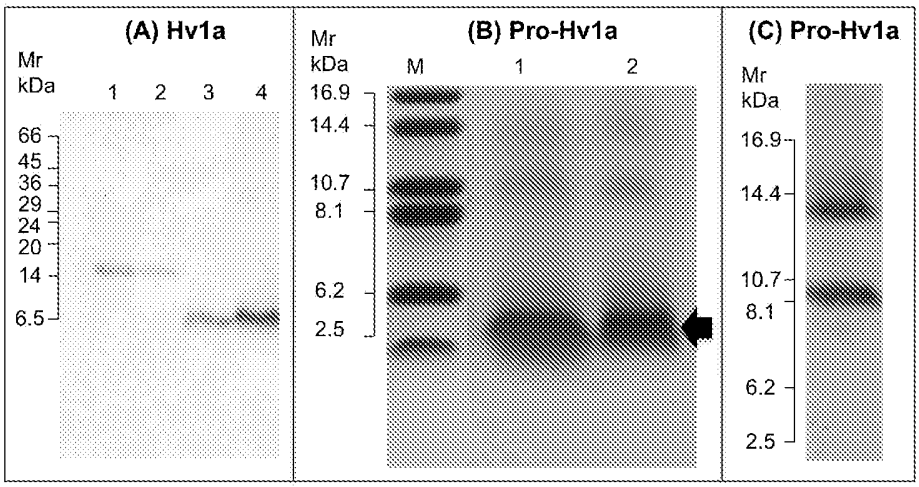


Figure 2: Expression and purification of recombinant Hv1a and pro-Hv1a toxin **(A)** SDS-PAGE gel (20% acrylamide) analysis showing purification of recombinant Strep-tagged Hv1a toxin from culture supernatant. Lanes 1 & 2 are GNA standards (0.5 & 0.25 µg, respectively) and lanes 3 & 4 are peak fractions (10 µl) following elution from Streptactin column with 2.5 mM desthiobiotin. **(B)** Tris-Tricine gel (15% acrylamide) analysis of recombinant pro-Hv1a, lanes 1 & 2 are peak fractions (10 µl) following elution from a nickel affinity column with 0.2 M imidazole. Arrow depicts major protein product predicted to be pro-Hv1a from which the histidine tag has been cleaved. **(C)** Western blot analysis of sample in (B) using anti-His antibodies.

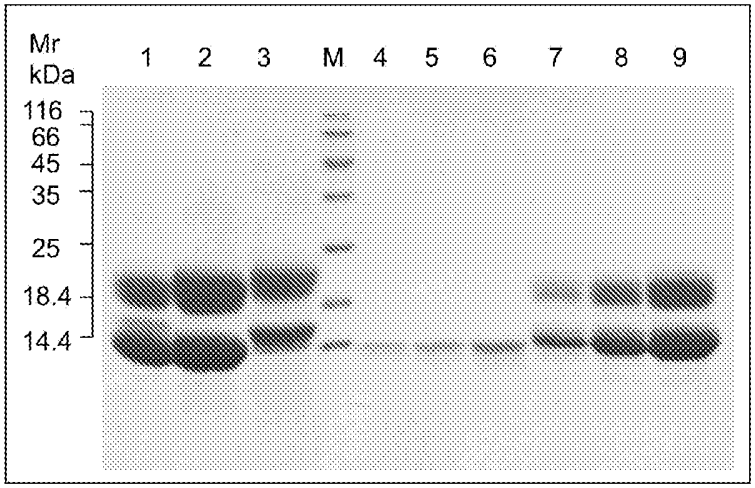
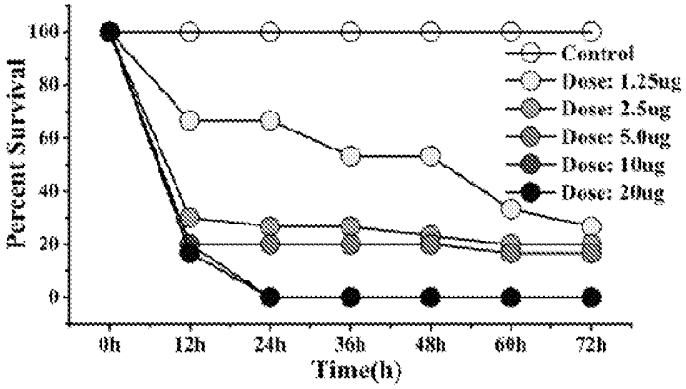
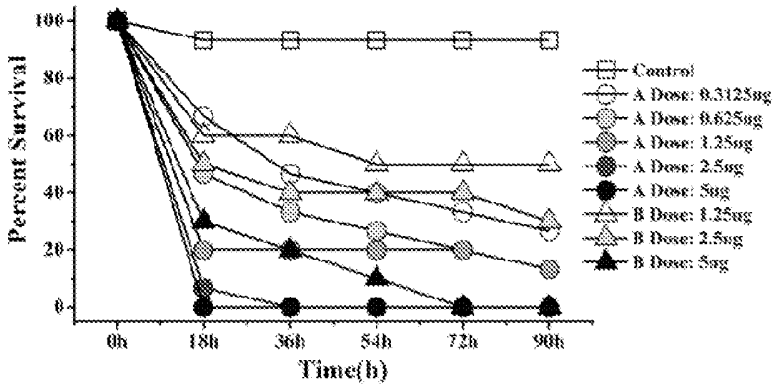


Figure 3: SDS-PAGE gel analysis of lyophilised samples of purified pro-Hv1a/GNA, Hv1a/GNA and MODHv1a/GNA. SDS-PAGE analysis (17.5% acrylamide gel) of purified recombinant Hv1a containing fusion protein, gel stained for total proteins with Coomassie Blue. Loading as follows: lane 1: pro-Hv1a /GNA; lane 2: Hv1a/GNA; lane 3: MODHv1a/GNA; lanes 4-6: GNA standards of 1, 2, & 4 μg, respectively; lanes 7-9: 12.5, 25, & 50 μg lyophilised purified pro-Hv1a/GNA (to enable quantification of fusion protein content).

(A) Pro-Hv1a 5th instar larvae



(B) Pro-Hv1a /GNA vs Hv1a/GNA 3rd to 4th instar larvae



(C) Pro-Hv1a /GNA vs Hv1a/GNA 5th instar larvae

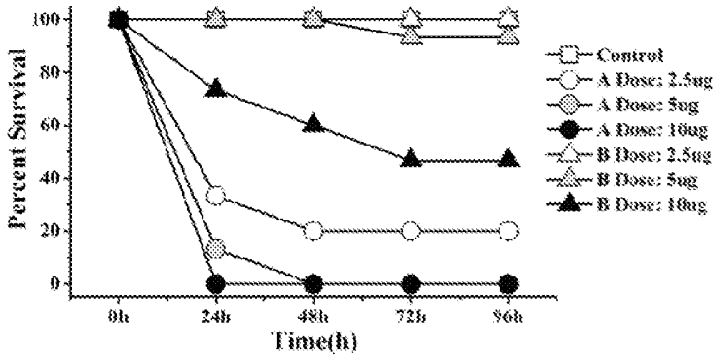


Figure 4: Injection toxicity of recombinant pro-Hv1a, pro-Hv1a/GNA and Hv1a/GNA to *Mamestra brassicae*

Percentage survival of 3rd-5th instar *Mamestra brassicae* larvae following the injection of different doses of recombinant pro-Hv1a, pro-Hv1a/GNA or Hv1a/GNA. **(A)** Percentage survival of 5th instar larvae following injection of various doses of pro-Hva1. **(B)** Percentage survival of 3rd-4th instar larvae following injection of various doses of pro-Hv1a/GNA (Dose A) or Hv1a/GNA (Dose B). **(C)** Percentage survival of 5th instar larvae following injection of various doses of pro-Hv1a/GNA (Dose A) and Hv1a/GNA (Dose B).

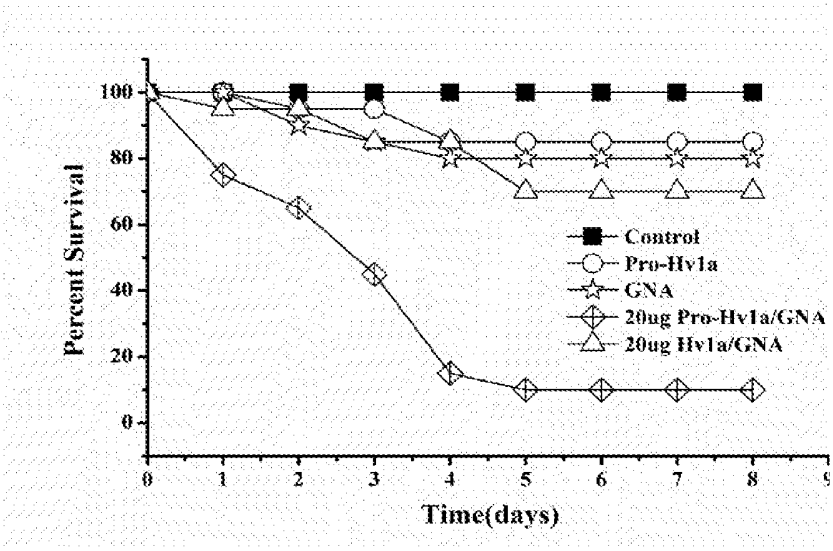
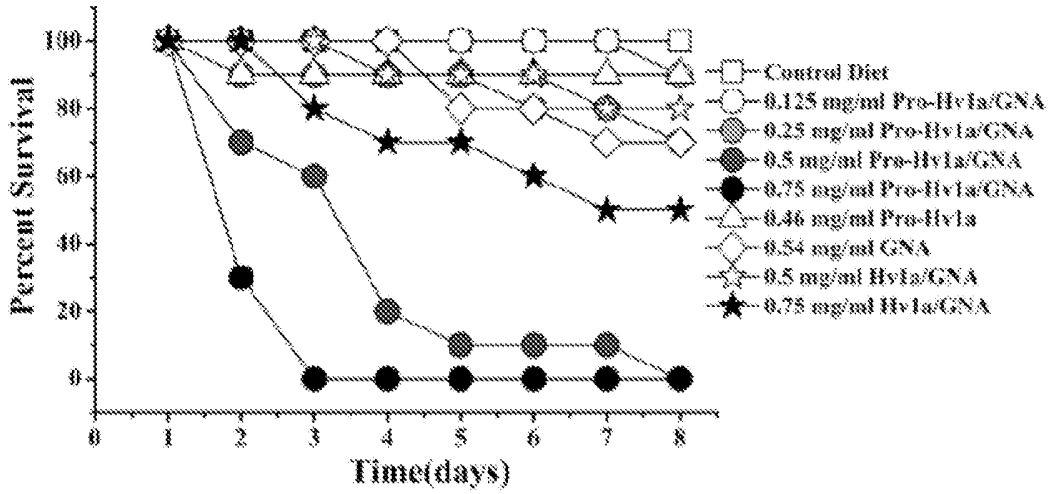


Figure 5: Ingestion toxicity of recombinant pro-Hv1a, Hv1a/GNA, pro-Hv1a/GNA and GNA to *Mamestra brassicae*. Percentage survival of 3rd instar *Mamestra brassicae* larvae following ingestion of a single 2 µl droplet containing 20 µg of purified pro-Hv1a, Hv1a/GNA, pro-Hv1a/GNA or GNA. Control larvae were fed on a droplet containing no added protein (n=10 per treatment).

(A)



(B)

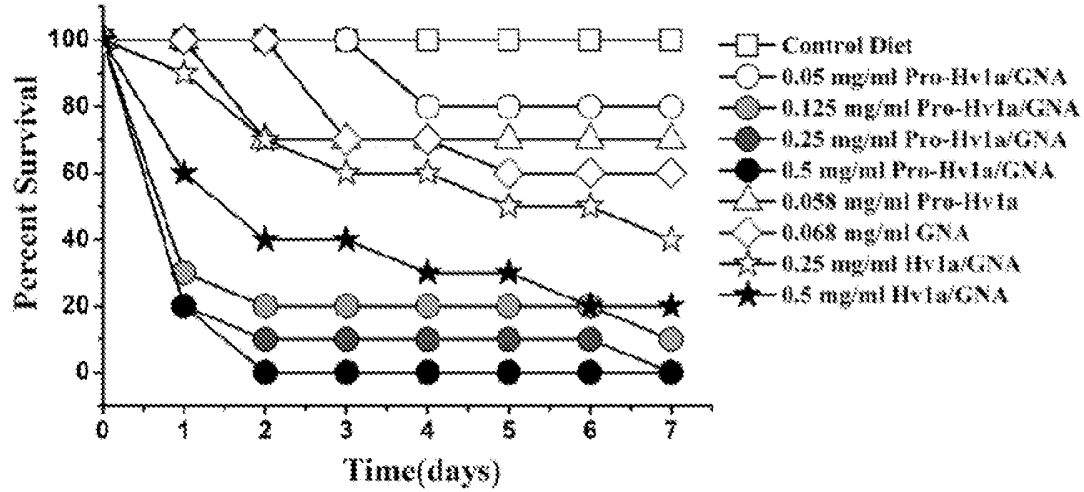


Figure 6: Ingestion toxicity of recombinant pro-Hv1a, pro-Hv1a/GNA, Hv1a/GNA or GNA to *Acyrthosiphon pisum* and *Sitobion avenae*

Percentage survival of **(A)** *Acyrthosiphon pisum* (pea aphids) and **(B)** *Sitobion avenae* (cereal aphids) with artificial diets containing 0.05 – 0.75 mg/ml purified recombinant pro-Hv1a, pro-Hv1a/GNA, Hv1a/GNA or GNA.

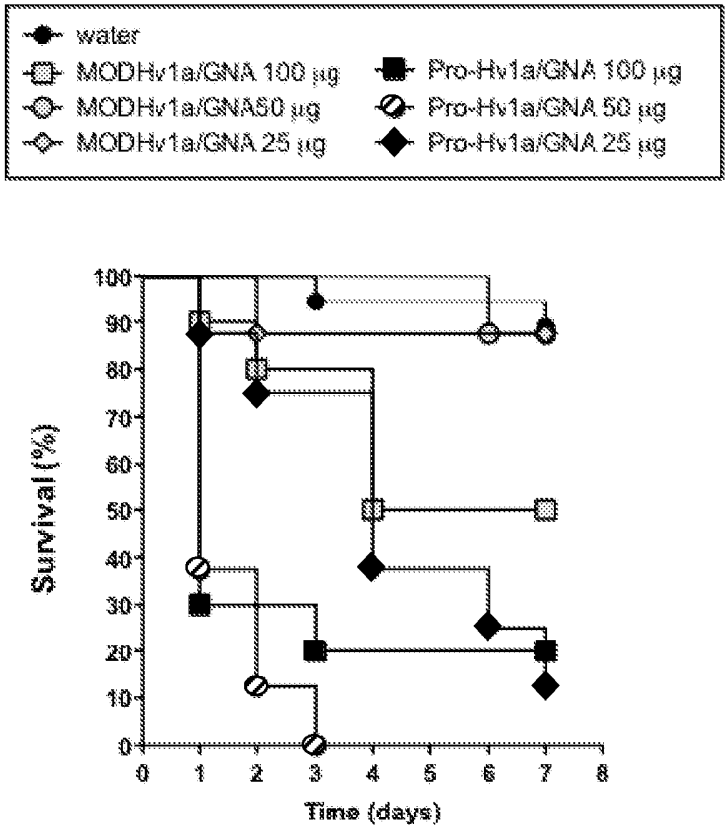


Figure 7: Injection toxicity of recombinant pro-Hv1a/GNA and Hv1a/GNA to *Deroceras reticulatum*

Percentage survival of *Deroceras reticulatum* (200 mg ± 40 mg) injected with 100, 50 or 25 µg of Hv1a/GNA or pro-Hv1a/GNA. n=18 for control treatment; n=10 for 100 µg dose and n=8 for 50 and 25 µg doses.

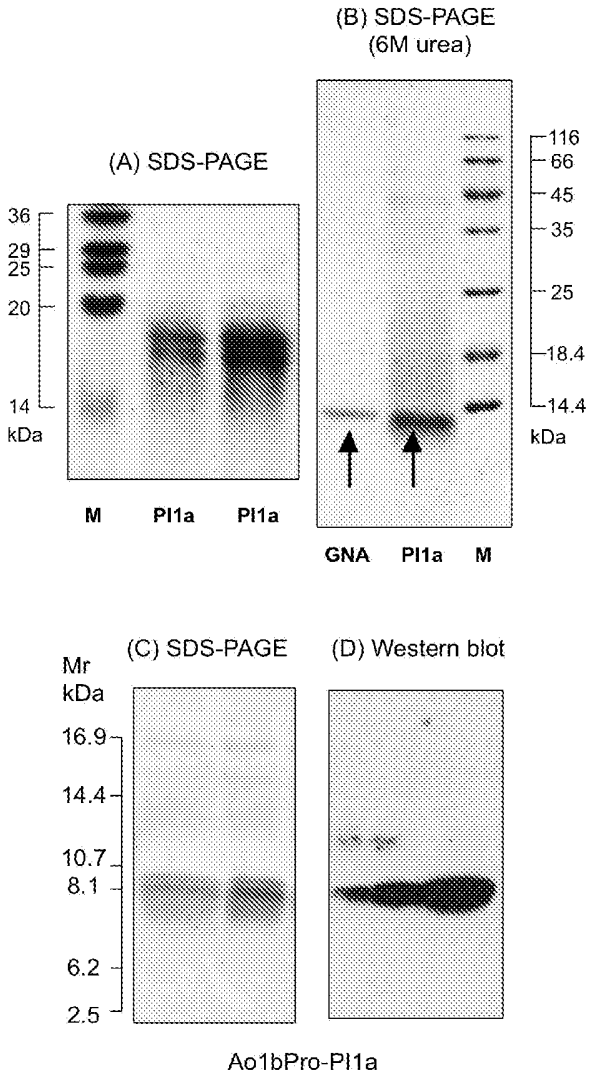


Figure 8: Expression and purification of recombinant Pl1a and Ao1bPro-Pl1a toxin

(A) Pl1a toxin derived from a construct encoding the mature toxin sequence separated on "normal" SDS-PAGE; M indicates marker, loadings of Pl1a are 5 and 10 µg. **(B)** Pl1a toxin (5 µg) separated on SDS-PAGE after denaturation by 6 M urea. **(C)** Recombinant Pl1a toxin derived from a construct containing the pro region designated Ao1b on SDS-PAGE, loadings of Ao1bPro-Pl1a are 2.5 µg. **(D)** Western blotting of purified Ao1bPro-Pl1a (25, 50 & 100 ng) using anti-His antibodies.

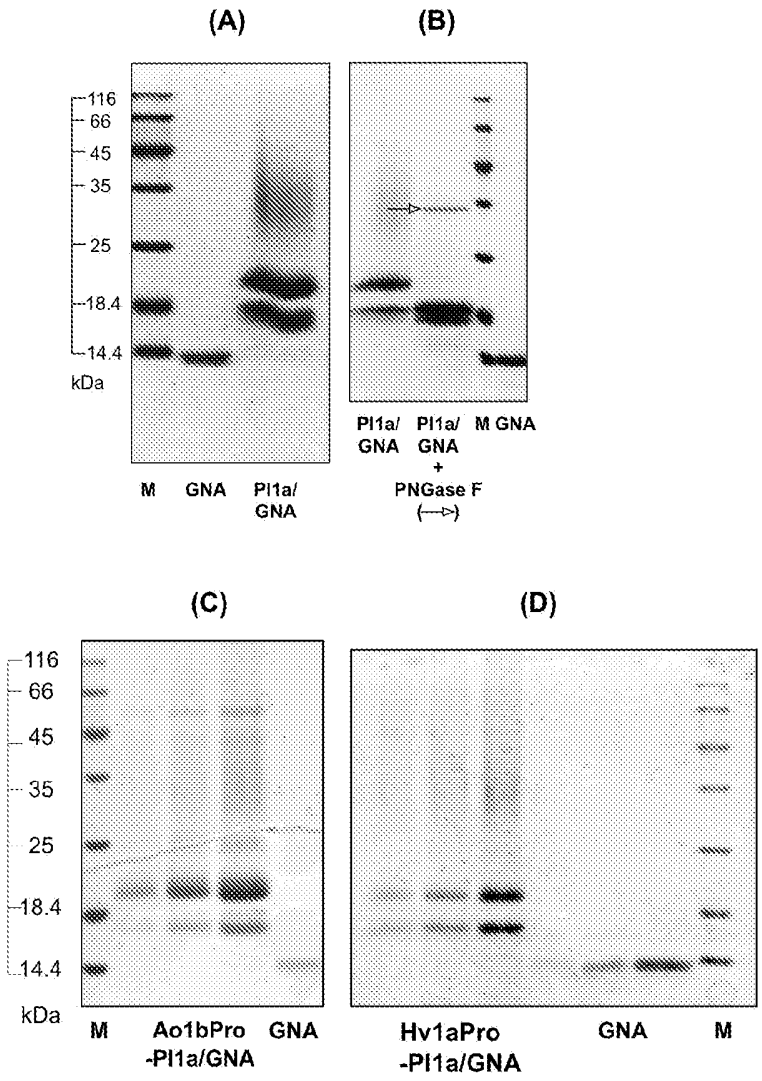
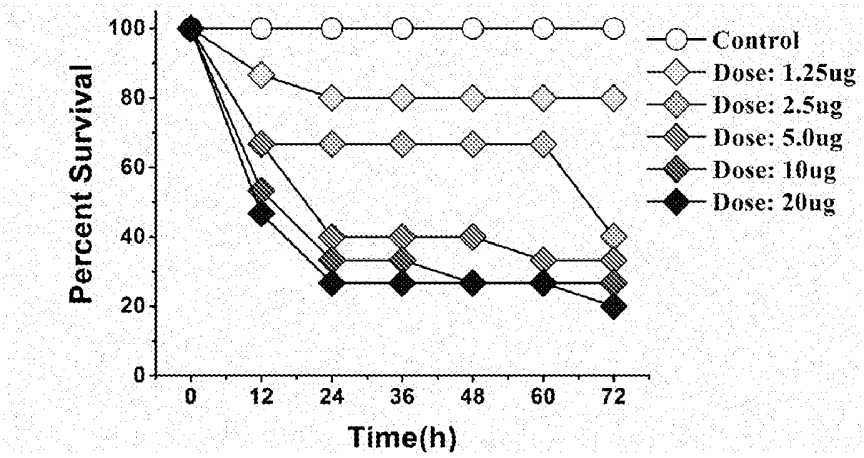


Figure 9: Characterisation of purified recombinant Pl1a/GNA fusion proteins by SDS-PAGE

(A) SDS-PAGE analysis of Pl1a/GNA fusion protein (10 µg) and GNA standard (5 µg). **(B)** Deglycosylation of Pl1a/GNA fusion protein using PNGase F (band indicated by open arrowhead), GNA standard (5 µg). **(C)** SDS-PAGE analysis of Ao1bPro-Pl1a/GNA (1, 2 and 4 µg), GNA standard (5 µg). **(D)** SDS-PAGE analysis of Hv1aPro-Pl1a/GNA (1, 2 and 4 µg), GNA standards (1, 2 and 4 µg).

(A) Pl1a injection



(B) Injection Pl1a and Ao1bPro-Pl1a – Dose A = Pl1a; Dose B = Ao1bPro-Pl1a

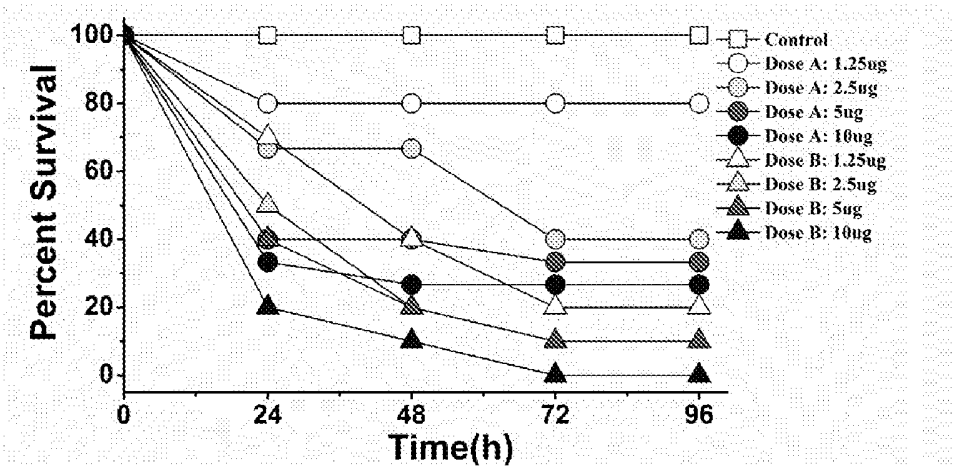
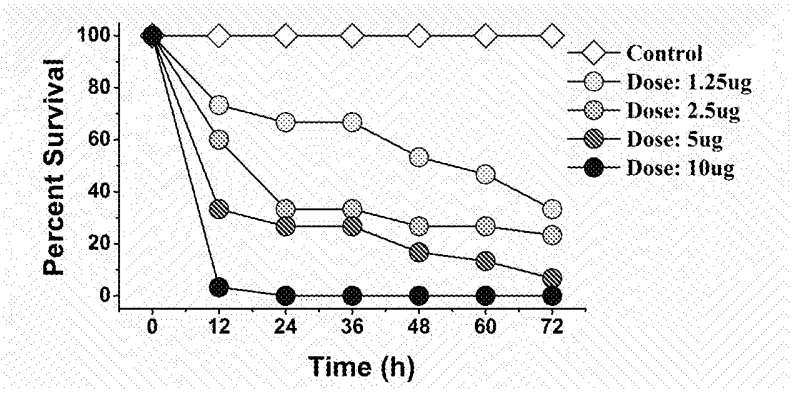


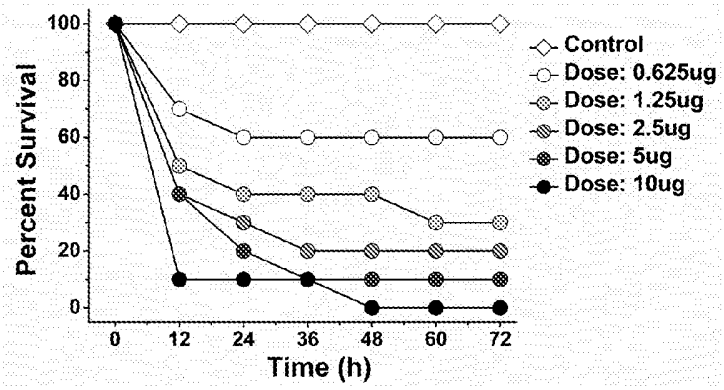
Figure 10: Injection toxicity of recombinant Pl1a and Ao1bPro-Pl1a to *Mamestra brassicae*

(A) Percentage survival of 5th instar *Mamestra brassicae* larvae following injection of different doses of purified recombinant Pl1a. **(B)** Percentage survival of 5th instar *Mamestra brassicae* larvae following injection of different doses of purified recombinant Pl1a (Dose A) or Ao1bPro-Pl1a (Dose B).

(A) Pl1a/GNA



(B) Ao1b-ProPl1a/GNA



(C) Pro-Hv1aPl1a/GNA

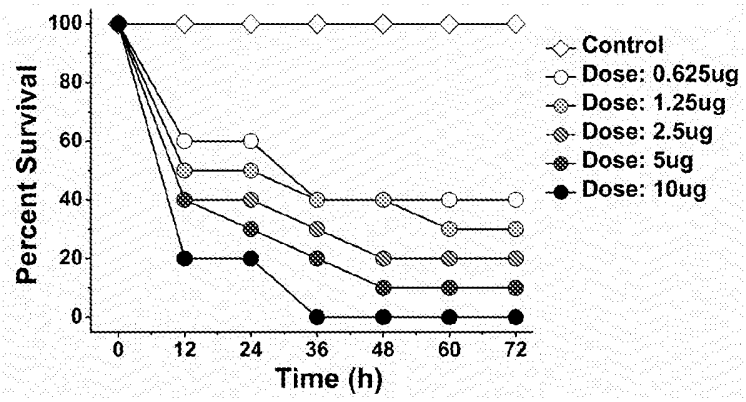
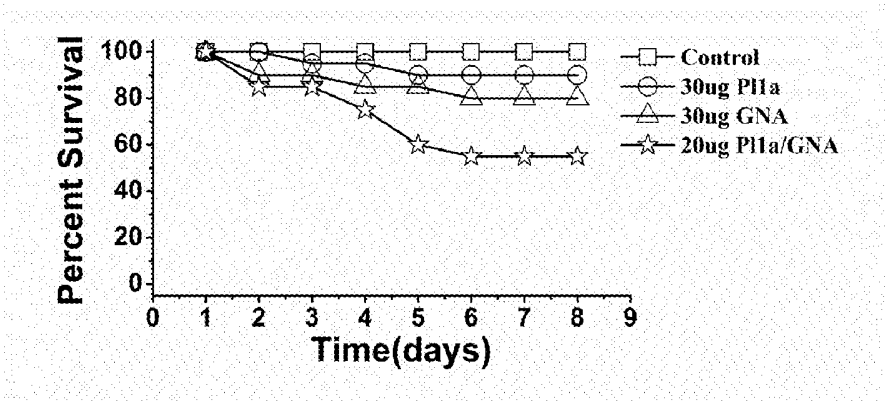


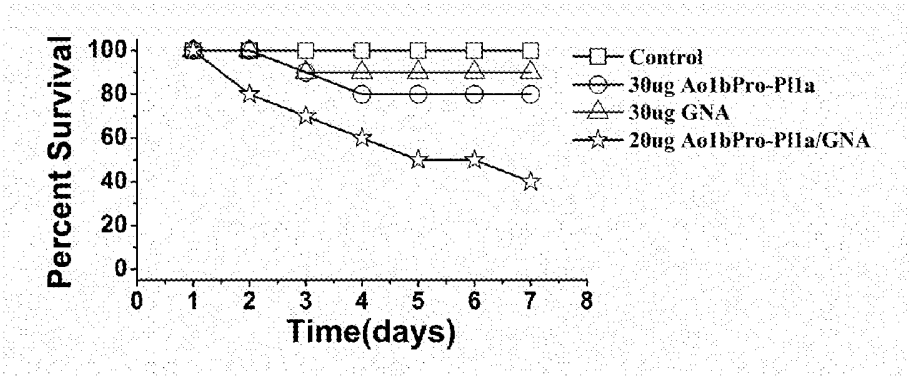
Figure 11: Injection toxicity of recombinant Pl1a/GNA, Ao1bPro-Pl1a/GNA and Pro-HV1a-Pl1a/GNA to *Mamestra brassicae*

Percentage survival of 5th instar *Mamestra brassicae* larvae following injection of different doses of purified recombinant Pl1a/GNA **(A)**, Ao1b-ProPl1a/GNA **(B)** or Pro-Hv1a-Pl1a/GNA **(C)**. n=20 per treatment.

(A) Droplet feeding: Pl1a/GNA



(B) Droplet feeding: Ao1bPro-Pl1a/GNA



(C) Droplet feeding: Hv1aPro-Pl1a/GNA

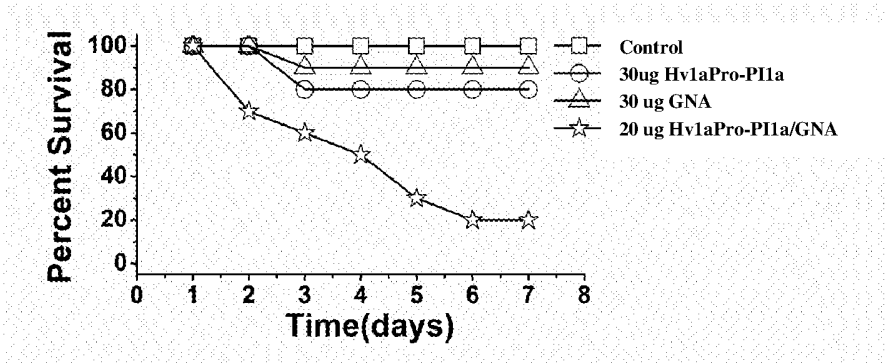


Figure 12: Ingestion toxicity of recombinant Pl1a/GNA, Ao1bPro-Pl1a/GNA and Hv1aPro-Pl1a/GNA to *Mamestra brassicae*

Percentage survival of 3rd instar *Mamestra brassicae* larvae following ingestion of a single 2 µl droplet containing 20 µg of purified Pl1a/GNA **(A)**, Ao1bPro-Pl1a/GNA **(B)** or Hv1aPro-Pl1a/GNA **(C)** fusion proteins. Controls in all cases were sucrose alone (no added protein), 30 µg of either Pl1a toxin (mature or modified form) or GNA.

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2014/053663

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/435
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/131302 A1 (UNIV DURHAM [GB]; FOOD AND ENVIRONMENT RES AGENCY FERA REPRESENTING TH) 4 October 2012 (2012-10-04) cited in the application	2-15, 17-34, 37-39
Y	the whole document	1
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
2 March 2015	09/03/2015

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2014/053663

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	Whole document, especially pages 44-58	1

X	WO 2013/026105 A1 (UNIV QUEENSLAND [AU]; KING GLENN FREDERICK [AU]; HARDY MARGARET CARTER) 28 February 2013 (2013-02-28)	2-34, 37-39
Y	Whole document, especially the claims	1

X	US 2007/066529 A1 (KING GLENN F [US] ET AL) 22 March 2007 (2007-03-22)	2-18, 25-34, 37-39
Y	Whole document, especially claims 3-7	1

X	US 2004/138423 A1 (KING GLENN F [US] ET AL) 15 July 2004 (2004-07-15)	2-18, 25-34, 37-39
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Y	EMILY S. W. WONG ET AL: "SVM-Based Prediction of Propeptide Cleavage Sites in Spider Toxins Identifies Toxin Innovation in an Australian Tarantula", PLOS ONE, vol. 8, no. 7, 22 July 2013 (2013-07-22), page e66279, XP055170991, DOI: 10.1371/journal.pone.0066279 abstract	1

A	CHONG ET AL: "The omega-atracotoxins: Selective blockers of insect M-LVA and HVA calcium channels", BIOCHEMICAL PHARMACOLOGY, ELSEVIER, US, vol. 74, no. 4, 12 July 2007 (2007-07-12), pages 623-638, XP022149874, ISSN: 0006-2952, DOI: 10.1016/J.BCP.2007.05.017 the whole document	1-39

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2014/053663

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SERGEY KOZLOV ET AL: "A novel strategy for the identification of toxinlike structures in spider venom", PROTEINS: STRUCTURE, FUNCTION, AND BIOINFORMATICS, vol. 59, no. 1, 1 April 2005 (2005-04-01), pages 131-140, XP055170851, ISSN: 0887-3585, DOI: 10.1002/prot.20390 the whole document ----	1-39
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2014/053663

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2009183282 A1	16-07-2009
		US 2011239331 A1	29-09-2011
		WO 2005025312 A2	24-03-2005

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 106414484 A
(43)申请公布日 2017.02.15

(21)申请号 201480075180.8
(22)申请日 2014.12.11
(30)优先权数据
1321938.1 2013.12.11 GB
(85)PCT国际申请进入国家阶段日
2016.08.09
(86)PCT国际申请的申请数据
PCT/GB2014/053663 2014.12.11
(87)PCT国际申请的公布数据
WO2015/087073 EN 2015.06.18
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代理人 单骁越
(51)Int.Cl.
C07K 14/435(2006.01)

权利要求书3页 说明书26页
序列表16页 附图12页

(54)发明名称

杀虫融合蛋白改进

(57)摘要

增加毒素的生物活性的方法。通过将前区域(pro-region)结合到用于产生杀虫剂毒素的核酸构建体中增加所述毒素的生物活性的方法。

CN 106414484 A

1.增加重组毒素的生物活性的方法,所述方法包括:

提供核酸构建体,所述核酸构建体包含:

(i)毒素序列或其片段或变体,所述毒素序列或其片段或变体与(ii)连接,

(ii)前区域或其片段或变体;和

任选地表达所述重组毒素。

2.核酸构建体,其包含:

(i)前区域或其片段或变体;和

(ii)与所述前区域相邻的位点,在所述位点中可以插入毒素基因序列或其片段或变体。

3.根据权利要求2所述的核酸构建体,其还包含插入在与所述前区域相邻的位点中的所述毒素基因序列或其片段或变体。

4.宿主细胞,其包含根据权利要求3所述的核酸构建体。

5.根据权利要求4所述的宿主细胞,其中所述细胞是酵母巴斯德毕赤酵母(Pichia pastoris)。

6.制备具有增加的生物活性的重组毒素的方法,所述方法包括

在适于表达所述重组毒素的条件下培养根据权利要求4或5所述的宿主细胞。

7.根据权利要求1或3-6中任一项所述的方法、核酸构建体或宿主细胞,其中所述毒素是杀虫剂毒素。

8.根据权利要求1或3-7中任一项所述的方法、核酸构建体或宿主细胞,其中所述毒素源自节肢动物、软体动物或其他无脊椎动物。

9.根据权利要求1或3-8中任一项所述的方法、核酸构建体或宿主细胞,其中所述毒素是节肢动物毒素,任选地,来自蓝山漏斗网蜘蛛(Hadronyche versuta)的ω-ACTX-Hv1a或κ-ACTX-Hv1c、来自阴暗拟隙蛛(Pireneitega luctuosus)的δ-amaurobitoxin-PI1a、管道网型蜘蛛(Segestria florentina)毒素Sf11-8、印度红蝎(Buthus mesotamulus)毒素ButaIT、来自Eucratoscelus constrictus的theraphotoxin Ec2a、来自Apomastus schlingeri的cyrtoautoxin As1a或来自Loxosceles intermedia的sicaritoxin Li1a。

10.根据权利要求1或3-9中任一项所述的方法、核酸构建体或宿主细胞,其中所述毒素包含20-100个氨基酸残基的肽。

11.根据权利要求1或3-10中任一项所述的方法、核酸构建体或宿主细胞,其中所述毒素是ω-ACTX-Hv1a或其片段或变体。

12.根据权利要求1或3-10中任一项所述的方法、核酸构建体或宿主细胞,其中所述毒素是δ-amaurobitoxin-PI1a或其片段或变体。

13.根据任一在前权利要求所述的方法、核酸构建体或宿主细胞,其中所述前区域是信号肽序列和成熟蛋白N-末端之间的序列,任选地其中所述前区域能够通过以下步骤鉴别:

将针对从其正常来源分离的蛋白所确定的序列与由编码其的基因预测的序列相比较;

鉴别不存在于最终蛋白产物中的N-端序列;

鉴别不存在于最终蛋白产物中的N-端序列中的信号肽序列;和

任选地通过从所述不存在于最终蛋白产物中的N-端序列移除所述信号肽序列,鉴别在所述信号肽序列和所述成熟蛋白N-末端之间的前区域序列。

14.根据任一在前权利要求所述的方法、核酸构建体或宿主细胞，其中所述前区域富含酸性氨基酸残基。

15.根据权利要求1、3-11、13或14中任一项所述的方法、核酸构建体或宿主细胞，其中在其天然存在的序列中所述毒素与所述前区域结合。

16.根据权利要求1或3-14中任一项所述的方法、核酸构建体或宿主细胞，其中在其天然存在的序列中所述毒素不与所述前区域结合。

17.根据任一在前权利要求所述的方法、核酸构建体或宿主细胞，其中所述前区域包含氨基酸序列EDTRADLQGGEAAEKVFRR或其片段或变体。

18.根据权利要求1至16中任一项所述的方法、核酸构建体或宿主细胞，其中所述前区域包含氨基酸序列ISYEEGKELFQKER或其片段或变体。

19.根据任一在前权利要求所述的方法、核酸构建体或宿主细胞，其中所述核酸构建体还包含载体蛋白序列或其片段或变体。

20.根据引用权利要求1或3-18中任一项的权利要求19所述的方法、核酸构建体或宿主细胞，其中所述载体蛋白的序列与所述毒素蛋白序列融合，由此在表达时产生融合蛋白。

21.根据权利要求19或20所述的方法、核酸构建体或宿主细胞，其中所述载体蛋白是凝集素。

22.根据权利要求19-21中任一项所述的方法、核酸构建体或宿主细胞，其中所述载体蛋白是选自以下各项的植物凝集素：雪花莲凝集素(GNA)、大蒜凝集素大蒜(Allium sativum)、豌豆凝集素豌豆(Pisum sativum)(P-lec)、花生凝集素花生(Arachis hypogaea)、菜豆凝集素(PHA,植物凝集素)或其片段或变体。

23.根据权利要求19-22中任一项所述的方法、核酸构建体或宿主细胞，其中所述载体蛋白是GNA。

24.核酸构建体，其包含：

(i)前区域或其片段或变体;和

(ii)载体蛋白序列或其片段或变体。

25.根据任一在前权利要求所述的方法、核酸构建体或宿主细胞，其中所述核酸构建体是表达构建体。

26.根据任一在前权利要求所述的方法、核酸构建体或宿主细胞，其中所述核酸构建体还包含编码亲和标签的序列。

27.根据权利要求26所述的方法、核酸构建体或宿主细胞，其中所述亲和标签是His-标签。

28.杀虫剂组合物，其包含根据权利要求1、6-23或25-27中描述的方法之一制备的毒素蛋白。

29.根据权利要求28所述的杀虫剂组合物，其中所述组合物为溶液、乳液、喷剂、混悬剂、粉剂、泡沫剂、糊剂、粒剂、气溶胶、胶囊或其他精细或粗略分开的材料或用于天然或合成材料的浸渍剂的形式。

30.根据权利要求28或29所述的杀虫剂组合物，其中所述组合物是与合适的稀释剂、佐剂、防腐剂、分散剂、溶剂和/或乳化剂混合的喷剂或混悬剂的形式。

31.根据权利要求28-30中任一项所述的杀虫剂组合物，其中所述组合物是与其他已知

杀昆虫剂、生长促进或调解物质、除草剂、杀真菌剂和/或增效剂在一起的紧密或物理混合物。

32.根据权利要求28-31中任一项所述的杀虫剂组合物，其中所述组合物包含的毒素蛋白的量为以重量计0.001％至99％，优选以重量计0.5％至98％，更优选以重量计1.0％至95％。

33.用于制备根据权利要求28-32中任一项所述的杀虫剂组合物的方法，所述方法包括将一定量的根据权利要求1、6-23或25-27中所述的方法之一制备的毒素蛋白与一种或多种合适的载体、稀释剂、佐剂、防腐剂、分散剂、溶剂、乳化剂以有效杀虫量混合。

34.预防或治疗植物的有害生物感染的方法，所述方法包括向所述植物或其生长地点施用一定量的根据权利要求1、6-23或25-27中描述的方法之一制备的毒素蛋白或根据权利要求28-32中任一项所述的杀虫剂组合物；或向所述植物引入根据权利要求3或引用权利要求3的权利要求7-23或25-27中任一项所述的核酸构建体。

35.根据权利要求34所述的方法，其中所述有害生物是软体动物有害生物，任选地是蛞蝓或蜗牛，任选地是麦蛞蝓。

36.杀软体动物诱饵组合物，其包含根据权利要求1、6-23或25-27中描述的方法之一制备的毒素蛋白和/或根据权利要求28-32中任一项所述的杀虫剂组合物。

37.转基因植物或其后代，所述转基因植物或其后代包含权利要求3或引用权利要求3的权利要求7-23或25-27中任一项中所述的核酸构建体，能够表达具有增加的生物活性的毒素。

38.根据权利要求3或引用权利要求3的权利要求7-23或25-27中任一项所述的核酸构建体或通过权利要求1、6-23或25-27中描述的方法之一制备的毒素蛋白在制备杀虫剂或转基因植物细胞或植物中的用途。

39.根据权利要求28-32中任一项所述的杀虫剂用于破坏或削弱一种或多种有害生物的用途。

杀虫融合蛋白改进

发明领域

[0001] 本发明涉及增加重组毒素的生物活性的方法。本发明还涉及含有前区域(pro-region)和毒素(尤其是节肢动物毒素)的序列的核酸构建体。

[0002] 发明背景

[0003] 针对不断增加的全球人口的背景,对变得越来越有效的食物生产系统的压力不断增加。尽管采取作物保护方面的进展,有害生物仍然是作物生产的主要限制。对最主要六种作物在世界范围内的可能损失的估计在25-80%之间变化(对于土豆40%)。一些有害生物和疾病可以通过施用农用化学品防治。然而,尽管在市场上可获得多种杀虫剂,植物病害仍是主要问题。

[0004] 在过去,大多数开发杀虫剂的研究集中于鉴别能够用于该目的的化学实体。然而,这些非靶特异的杀虫剂常常导致环境破坏,并且对非靶向物种(包括动物物种)和人健康具有消极影响。因此,已经批准禁止将某些化学化合物用于杀虫剂的欧盟立法。因此,已经向鉴别可以用于有害生物控制的新类型的"生物杀虫剂"转变。生物杀虫剂通常被认为是天然存在的物质(生化杀虫剂),能够防治有害生物的微生物(微生物杀虫剂)和含有添加的遗传物质的植物生产的杀虫物质(植物结合的防护剂)。希望该开发生物杀虫剂的驱动力将导致用于预防植物病害的更环境友好的选择。

[0005] 由蜘蛛和其他节肢动物作为毒液合成的神经肽毒素已经是用于作为生物杀虫剂开发的研究的主题。WO2006/052806、WO2005/025313和US2007/0066529描述了蜘蛛毒素毒液肽用作生物杀虫剂的用途,并且Khan等人,2006描述了在植物中表达蜘蛛毒液毒素以保护植物免受昆虫攻击。本发明人之前表明,ω-ACTX-Hv1a,一种源自蓝山漏斗网蜘蛛(Hadroncyhe versuta)的毒素,当融合能够介导融合蛋白自无脊椎动物消化道(gut)的移位的蛋白如雪花莲凝集素"载体"GNA时,可以用作针对宽范围有害生物的有效杀虫剂(WO2012/131302)。

[0006] 发明概述

[0007] 本发明部分基于本发明人对在用于表达重组毒素的构建体中包含前区域对所述重组毒素的生物活性的影响的研究。

[0008] 本研究者希望确定可以如何提高体外表达的重组毒素蛋白的生物活性。为了研究这一点,本发明人分析了编码节肢动物毒素的基因的DNA序列。在WO2012/131302中使用的节肢动物毒素是小的、富含半胱氨酸的蛋白,其属于蛋白序列的几个超家族(其包括来自除了节肢动物之外的生物的毒素)。所述编码基因包括两种在最终蛋白产物中不存在的序列;在翻译期间移除的预测的N-端信号肽和在信号肽和如分离的蛋白的最终序列之间的预测的前区域(参见图1A)。前区域是节肢动物和其他生物中的小肽毒素的常见特征(Windley等人,2012)。本发明人出人意料地发现在用于表达重组毒素的构建体中包含该预测的前区域导致比由缺少前区域的构建体制备的毒素更好的生物活性。此外,与由缺少前序列的构建体制备的毒素相比,在用于表达在基因组DNA序列中并不天然含有前序列的毒素(例如δ-amaurobitoxin-PI1a)的构建体中包含前序列同样导致增加的生物活性。

[0009] 因此，在本发明的第一方面，提供增加重组毒素的生物活性的方法，所述方法包括：

[0010] 提供核酸构建体，所述核酸构建体包含：(i)毒素序列或其片段或变体，所述毒素序列或其片段或变体连接于(ii)前区域或其片段或变体；和任选地表达重组毒素。

[0011] 在本发明的第二方面，提供核酸构建体，其包含：(i)前区域或其片段或变体；和(ii)与所述前区域相邻的位点，其中可以插入毒素基因序列或其片段或变体。

[0012] 在根据本发明的第二方面的实施方案中，所述核酸构建体还包含插入在与所述前区域相邻的位点中的毒素基因序列或其片段或变体。

[0013] 在本发明的第三方面中，提供宿主细胞，其包含根据本发明的第二方面的其中插入有毒素基因序列的核酸构建体或其任意实施方案。

[0014] 在本发明的第四方面中，提供制备具有增加的生物活性的重组毒素的方法，所述方法包括在适于表达重组毒素的条件下培养本发明的第三方面中所述的宿主细胞。

[0015] 根据本发明的上述方面的毒素是杀虫剂毒素。在本发明的优选的实施方案中，毒素可以源自节肢动物、软体动物或其他无脊椎动物。

[0016] 在本发明的一个实施方案中，所述毒素是节肢动物毒素。本发明的节肢动物毒素可以包括来自蓝山漏斗网蜘蛛(Hadronyche versuta)的ω-ACTX-Hv1a和κ-ACTX-Hv1c、来自阴暗拟隙蛛(Pireneitega luctuosus)的δ-amaurobitoxin-PI1a、管道网型蜘蛛(Segestria florentina)毒素Sf11-8、印度红蝎(Buthus mesotamulus)毒素ButaIT、来自Eucratoscelus constrictus的theraphotoxin Ec2a、来自Apomastus schlingeri的cyrtoautoxin As1a、来自Loxosceles intermedia的sicaritoxin Li1a，和其他类似毒素。

[0017] 根据本发明的毒素可以包含20-100个氨基酸残基的肽。所述毒素可以含有多个半胱氨酸残基，其形成内部二硫键。

[0018] 在一个实施方案中，所述毒素是ω-ACTX-Hv1a或其片段或变体。ω-ACTX-Hv1a毒素是本领域中已知的(Fletcher等人，1997)。其是分离自蓝山漏斗网蜘蛛(Hadroncyhe versuta)的毒素。ω-ACTX-Hv1a的氨基酸序列是已知的，编码ω-ACTX-Hv1a的核酸序列也是已知的。ω-ACTX-Hv1a毒素是钙通道拮抗剂，其之前已被证明阻断无脊椎动物钙通道，但不阻断脊椎动物钙通道。在多数情况下，希望使用对脊椎动物没有活性的杀虫剂，从而避免对人或动物的有害作用。

[0019] 之前报道ω-ACTX-Hv1a在局部用于毛虫时其本身可以用作杀虫剂(Khan等人，2006)。然而，上述文献的作者报道的是所述肽在含有已知自身具有杀昆虫性的咪唑的溶液中的局部应用。此外，未报道单独的所述肽的杀昆虫活性的进一步证据，其他涉及ω-ACTX-Hv1a的公开仅述及通过注射入无脊椎动物有害动物的活性。

[0020] 本发明人之前已经证明，重组ω-ACTX-Hv1a的生物活性可以通过产生融合蛋白来提高，其中毒素ω-ACTX-Hv1a与能够介导融合蛋白自无脊椎动物消化道的移位的“载体”肽融合(WO2012/131302)。本发明人使用植物凝集素GNA作为这样的载体肽的实例。

[0021] 为了研究可以如何另外地提高重组毒素融合蛋白的生物活性，本发明人分析了编码节肢动物毒素尤其是ω-ACTX-Hv1a的基因的DNA序列。发现很多节肢动物基因含有不存在于最终蛋白中的预测的前区域，其之前未被结合到用于体外表达融合蛋白的构建体中。如可从本文看出的，本发明人将前区域的序列结合到用于制备重组毒素蛋白的核酸构建体

中。本发明人发现，与由未修饰的构建体(即含有ω-ACTX-Hv1a序列而没有另外的前区域)制备重组ω-ACTX-Hv1a相比，通过注射或在包含在膳食中的情况下施用于多种无脊椎有害生物的由含有前区域的构建体制备的重组ω-ACTX-Hv1a导致增加的瘫痪和死亡。因此，当以此形式提供时，ω-ACTX-Hv1a肽毒素可以非常有效地作为针对无脊椎动物的杀虫剂。

[0022] 如本文使用的，并且如下文进一步说明的，“杀虫剂”是指针对任何有害生物使用的化学物质、生物剂(如病毒或细菌)、抗微生物剂、消毒剂或装置。有害生物包括昆虫、植物病原体、杂草、软体动物、鸟、哺乳动物、鱼、线虫(蛔虫)和破坏财产、传播疾病或作为疾病的载体或引起麻烦的微生物。然而，对于本发明，通过“杀虫剂”我们是指有害生物是任何破坏财产，尤其是农产品的无脊椎动物。

[0023] 在备选实施方案中，本发明可以包含δ-amaurobitoxin-PI1a毒素或其片段或变体。

[0024] 毒素δ-amaurobitoxin-PI1a来自蜘蛛阴暗拟隙蛛(Pireneitega luctuosus)并且在其内源基因序列中不含有预测的前区域。出人意料地，本发明人发现，在用于重组δ-amaurobitoxin-PI1a的表达构建体中包括前区域(基于存在于全球数据库中的类似序列设计)产生针对无脊椎有害生物具有增加的生物活性的重组毒素。该意料之外的研究结果表明，可以使用本发明通过设计前区域并将其结合到表达构建体中来增加不含有与其内源基因序列结合的前区域的重组毒素的生物活性。

[0025] 尽管不希望受理论限制，但是据信，在核酸构建体中包括前区域，导致毒素在体外表达时折叠的改善。

[0026] “片段或变体”包括的是，本发明的毒素序列可以与天然存在的序列不同，条件是所述片段或变体基本上保持毒素的生物活性。保持毒素的生物活性意为与天然毒素相比，所述片段和/或变体保持至少部分的杀虫剂活性。通常，所述片段和/或变体保持至少50％，如60％、70％、80％或90％活性。在一些情况下，所述片段和/或变体可以具有比天然毒素更高的杀虫剂活性。在一些实施方案中，与天然毒素相比，所述片段和/或变体可以显示另一种生理特征的提高。例如，与天然毒素相比，所述片段和/或变体可以具有更长的体外和/或体内半衰期。

[0027] 序列的“变体”包括的是保守或非保守插入、缺失和置换。尤其包括这样的核苷酸序列变体，其中此种改变基本上不改变毒素的生物活性。技术人员将知道可以在损失生物活性的情况下改变这样的序列。特别地，核苷酸序列的单个改变可以不导致序列表达后氨基酸序列的改变。此外，如果核苷酸序列的改变导致选择性的氨基酸的结合，但其中相应的氨基酸的理化性质基本上没有改变(例如，保守置换如Gly，Ala；Val，Ile，Leu；Asp，Glu；Asn，Gln；Ser，Thr；Lys，Arg；和Phe，Tyr)，则相应的毒素的功能性不应该受影响。此外，毒素的非功能区域内的小的缺失也是可容忍的，并且因此被认为是用于本发明的“变体”。“变体”还包括重组毒素蛋白，其中氨基酸通过例如糖基化或二硫键形成被翻译后修饰。技术人员可以容易地采用本文中描述的实验过程来确定“变体”是否仍然可以作为毒素行使功能。

[0028] 如果变体的序列与毒素的“天然存在的”核苷酸序列具有至少75％，进一步更优选至少80％，进一步优选至少85％，更进一步优选至少90％和最优选至少95％或97％同一性，则是优选的。

[0029] 在优选的实施方案中，本发明涉及节肢动物毒素。本发明人研究的节肢动物毒素

是小的、富含半胱氨酸的蛋白，其属于多个蛋白序列超家族。这些毒素中的一些的基因序列含有不存在于最终蛋白产物中的序列。这些额外的序列包括在翻译期间被移除的预测的N-端信号肽和在信号肽与如分离的蛋白的最终序列之间的预测的前区域(参见图1A)。

[0030] 多数富含半胱氨酸的小肽毒素最初被翻译为较大的前体(70-120个氨基酸)，所述前体含有在翻译期间被移除的保守的N-端信号肽(大约20个氨基酸的)，在蛋白家族内显示显著序列保守性的前区域(大约15-60个氨基酸)，和产生成熟毒素并且更可变的C-端毒素编码区。

[0031] 前区域已经从编码很多毒素的cDNA序列被预测，并且当在蛋白家族的成员之间比较时其通常不如成熟毒素序列可变，尽管还未鉴别序列基序如在信号肽中发现的那些。然而，前区域常常富含酸性氨基酸残基(Tedford等人，2004)。例如，与ω-ACTX-Hv1a毒素相关的前区域的氨基酸序列是：

[0032] EDTRADLQGGEAAEKVFRR(还参见图1B)

[0033] 与Ao1b毒素相关的前区域的氨基酸序列是：

[0034] ISYEEGKELFQKER

[0035] 前区域可以通过将对从其正常来源分离的蛋白确定的序列与通过编码它的基因预测的序列相比较来鉴定。这样的比较可以显示蛋白水解或裂解步骤是否与转录同时或在转录后发生从而获得最终蛋白产物。前区域被从成熟蛋白的N-末端移除；然而，它们区别于参与导向多肽进入分泌途径的信号肽。信号肽可以使用软件算法如SignalP*(Nielsen等人，1997)基于通过基因预测的蛋白序列鉴定。为了鉴别前区域，首先将直接确定的蛋白序列与预测的序列相比，以显示一个区域被从N-末端移除；；然后通过从所述软件预测确定信号肽的存在；然后前区域可以被鉴定为信号肽和成熟蛋白N-末端之间的序列区域。可以基于上述理念，使用可在Arachnoserver数据库(http://www.arachnoserver..org/spiderP.html；Wong等人，2013)上自由获得的软件(SpiderP)预测节肢动物毒素中的前区域。该支持向量机(SVM)法使用专门设计的算法来将局域和全球序列信息相结合。

[0036] 使用上述方法鉴别的前区域可以用于本发明。在其天然存在的序列中本发明的毒素可以与本发明的上述方面的前区域结合。备选地，前区域的序列可以基于可在全球数据库中获得的序列设计，或基于上述方法鉴定，并且结合到通常(即，在天然存在的序列中)不与前区域结合或在天然存在的序列中与不同的前区域结合的毒素的核酸构建体中。

[0037] 在本发明的实施方案中，前区域包含氨基酸序列EDTRADLQGGEAAEKVFRR或其片段或变体。

[0038] 在本发明的进一步实施方案中，前区域包含氨基酸序列ISYEEGKELFQKER或其片段或变体。

[0039] 前区域的“片段或变体”包括的是，前区域的核酸序列可以不同于本领域中已知的和天然存在的，条件是所述片段或变体基本上保持前区域的生物活性，即其仍然能够提高与其相结合的毒素的生物活性。

[0040] 在伴随的实施例中，本发明人已经表明，前区域可以在表达期间被去除，以致其不存在于最终蛋白或融合蛋白中。然而，应该理解，表达后保持前区域的蛋白/融合蛋白仍落在本发明的范围内。

[0041] 根据本发明的上述方面的核酸构建体和其任意实施方案也可以含有能够介导由

构建体产生的蛋白自无脊椎动物消化道的移位的蛋白(“载体”蛋白)的序列或其片段或变体。该序列可以融合至毒素蛋白序列,从而产生融合蛋白。任何结合于昆虫消化道的蛋白都可以用作载体蛋白,条件是其在消化道中发现的环境下稳定,并且对哺乳动物无毒性。

[0042] 能够用作载体蛋白的合适的蛋白包括凝集素。通常,可以使用任何结合于昆虫消化道的凝集素。在本发明的一个实施方案中,载体蛋白是植物凝集素。

[0043] 本发明人之前证明某些植物凝集素对消化道蛋白水解有抗性并且有能力充当载体以将其他肽从靶物种的消化道递送到循环系统。本发明人还证明将植物凝集素与毒素融合有助于跨无脊椎有害生物的消化道壁的移位,由此增加毒素的生物活性,并且使得这样的融合蛋白能够被用作杀虫剂。

[0044] 本发明的优选实施方案是这样的,其中所述载体蛋白是选自以下各项中的任一种或多种的植物凝集素:雪花莲凝集素(GNA)、大蒜凝集素大蒜(Allium sativum)、豌豆凝集素豌豆(Pisum sativum)(P-lec)、花生凝集素花生(Arachis hypogaea)、菜豆凝集素(PHA,植物凝集素)或其片段或变体。

[0045] 植物凝集素的“片段或变体”包括的是,特定凝集素的核酸序列可以区别于本领域中已知的和天然存在的,条件是所述片段或变体基本上保持凝集素的生物活性,即其能够介导融合蛋白自无脊椎动物消化道的移位。

[0046] 在优选的实施方案中,所述凝集素是GNA。在伴随的实施例中,本发明人出人意料地证明,与单独的毒素、仅与GNA连接的毒素和仅与前区域连接的毒素相比,由除了前区域和GNA的序列外还含有毒素序列的核酸构建体制备的毒素蛋白产生针对无脊椎有害生物的增加的生物活性。虽然预期添加前区域或与另一蛋白融合能够各自增强重组毒素的生物活性,但是不会预期两种修饰的组合将产生加成效应。考虑到的是这两种修饰都会导致相同的结果,即正确的蛋白折叠,并且因此任一种修饰或二者会导致生物活性的相同的增强。本发明人已经证明这并非事实。

[0047] 如将理解的,用于制备包含(i)节肢动物毒素序列,(ii)前区域和(iii)载体蛋白序列的核酸构建体的起始材料是由(i)前区域和(ii)载体蛋白序列组成的核酸构建体。

[0048] 因此,本发明的第五方面包括核酸构建体,所述核酸构建体包含(i)前区域或其片段或变体和(ii)能够介导由所述构建体生产的蛋白自无脊椎动物消化道的移位的蛋白(载体蛋白)的序列或其片段或变体。

[0049] 前区域和载体蛋白可以是关于本发明的上述方面所讨论的任何前区域/载体蛋白。

[0050] 制备核酸构建体的方法使用常规分子生物学技术。已经开发了多种方法用于连接多核苷酸以形成连续的单链或双链(特别是双链)DNA,例如经由利用限制性酶的消化产生的互补粘性末端。合适的方法在Sambrook&Russell,Molecular Cloning:A Laboratory Manual:第3版中描述。技术人员可以容易地使用这样的方法来制备根据本发明第二方面的核酸分子。此外,伴随的实施例提供了关于如何制备这样的分子的进一步详情。

[0051] 希望的制备本发明的核酸构建体的方法使用聚合酶链式反应。该方法可以用于例如通过改造限制性酶消化的合适位点将DNA引入合适的载体中,或其可以用于以本领域中已知的其他有用方式修饰DNA。

[0052] 在本发明的实施方案中,根据本发明的上述方面及其实施方案的核酸构建体是表

达构建体。

[0053] “表达构建体”是本领域中公知的术语。表达构建体是生物技术中用于生产重组蛋白的基本工具。表达构建体通常包括用于将具体核酸序列引入靶细胞“宿主细胞”中的质粒。一旦表达构建体在细胞内部，由该核酸序列编码的蛋白由细胞转录和翻译机制核糖体复合物生产。所述质粒还可以包括维持和扩增载体所需的核酸序列(在一些情况中通过整合到宿主基因组中)。表达载体的目标是生产大量的稳定的信使RNA，并且因此生产蛋白。

[0054] 本发明的核酸构建体还可以包含适当的调节序列，其包括启动子序列、终止子片段、增强子序列、标志物基因和/或其他序列。对于进一步详情，参见，例如，Sambrook& Russell，Molecular Cloning:A Laboratory Manual:第3版。

[0055] 还可以将核酸构建体改造成含有充当增强子和启动子区的调节序列并且导致所述构建体上携带的融合蛋白序列的有效转录。调节单元的很多部件位于异源基因的编码序列的上游并且与其可操作连接。如果预期在真核宿主例如巴斯德毕赤酵母(Pichia pastoris)中表达，则所述核酸构建体还可以含有包含聚腺苷酸化位点的下游3’非翻译区。调节序列可以引导异源编码序列的组成型或诱导型表达。

[0056] 用于连接个体核酸片段以产生本发明的核酸构建体的方法可以在前区域的N-末端引入2-4个额外的氨基酸残基，并且在载体蛋白的C-末端引入多至12个氨基酸。在毒素和载体序列间还可以存在短的“接头”区。这些另外的氨基酸残基保持编码序列并且不影响毒素或融合蛋白的活性。

[0057] 如本文使用的，术语“生物活性”是指重组毒素对无脊椎有害生物的毒性。生物活性的计算可以基于LD_{50}。

[0058] 与由没有前区域的核酸构建体生产的重组毒素的生物活性相比，将前区域结合入用于表达重组毒素的核酸构建体可以导致生物活性的至少25％，50％，100％，200％，300％，400％或更高的增加。在本情况中，这样的生物活性可以是杀虫剂活性，其可以通过多种技术测量，包括有害生物死亡、减少的寿命、繁殖限制如减少的繁殖率或卵产生等。

[0059] 与由含有毒素序列和前区域的核酸构建体生产的重组毒素的生物活性相比，进一步将载体蛋白的序列结合入用于表达重组毒素的表达构建体可以导致生物活性的至少25％，50％，100％，200％，300％，400％或更高的增加。

[0060] 本发明的表达系统可以是原核的或真核的。合适的表达系统包括细菌表达系统(例如大肠杆菌(E.coli)和枯草芽胞杆菌(Bacillus subtilis))，酵母表达系统(例如酿酒酵母(Saccharomyces cerevisiae)和巴斯德毕赤酵母)，丝状真菌表达系统(例如曲霉(Aspergillus))，和植物、动物和昆虫细胞表达系统。然而，优选的是，使用的表达系统是巴斯德毕赤酵母。毕赤酵母蛋白表达系统是本领域中公知的，并且因此可容易地获得用作宿主细胞的细胞。

[0061] 本发明的宿主细胞可以是原核或真核的。优选的原核宿主细胞通常是大肠杆菌菌株如，例如大肠杆菌菌株DH5和RR1。优选的真核宿主细胞包括酵母、昆虫和哺乳动物细胞，优选脊椎动物细胞如来自小鼠、大鼠、猴或人成纤维细胞系的那些。酵母宿主细胞包括YPH499、YPH500和YPH501，其通常可从Stratagene Cloning Systems，La Jolla，CA92037，USA获得。然而，优选的是，宿主细胞是酵母巴斯德毕赤酵母。毕赤酵母蛋白表达系统是本领域中公知的，并且因此可容易获得用作宿主细胞的细胞。特别优选的是，细胞株是

SMD1168H,其可以从Invitrogen™获得。

[0062] 用核酸构建体转化适当细胞宿主通过已知方法完成,所述方法通常取决于使用的载体的类型。关于原核宿主细胞的转化,参见例如,Sambrook&Russell,Molecular Cloning:A Laboratory Manual:第3版。酵母细胞的转化描述于Sherman等人,1986中。

[0063] 电穿孔也可用于转化和/或转染细胞,并且在本领域中公知用于转化酵母细胞、细菌细胞、昆虫细胞和脊椎动物细胞。通过电穿孔转化酵母的方法在Becker&Guarente,1990中公开。

[0064] 成功转化的细胞,即含有根据本发明的核酸构建体的细胞可以通过公知技术鉴别。例如,可以将通过引入本发明的核酸构建体得到的细胞培养以产生毒素融合蛋白。可以将细胞收获并裂解,并且针对所述DNA的存在检查其DNA内含物,如Southern,1975或Berent等人,1985描述的。

[0065] 因此,除了转化的宿主细胞本身,本发明还预期在营养培养基中的所述细胞的培养物,优选单克隆培养物,或源自单克隆培养物的培养物。

[0066] 本发明的上述方面的一个实施方案是这样的,其中核酸构建体还包含编码亲和标签的序列以帮助在表达后回收和纯化毒素蛋白。

[0067] 使用短氨基酸标签序列来帮助亲和纯化重组蛋白是本领域中公知的。事实上,很多可商购的蛋白表达构建体包括编码这样的标签的核酸序列。将目的蛋白以这样的方式插入表达构建体中,使得亲和标签与所述蛋白相连。多种不同的亲和标签在本领域中是已知的,包括几丁质结合蛋白(CBP),麦芽糖结合蛋白(MBP),谷胱甘肽-S-转移酶(GST),和聚组氨酸-标签(His-标签)。

[0068] His-标签是蛋白中的氨基酸基序,其由至少五个组氨酸(His)残基组成,常常在蛋白的N-或C-末端。其也被称为六组氨酸-标签,6xHis-标签,并且商标名为His-tag®。其是公知的亲和标签并且将His-标签引入重组蛋白的方法是本领域中已知的,纯化具有His-标签的蛋白的常规方法也是已知的。本发明的优选实施方案是这样的,其中另外的亲和标签序列编码His-标签。

[0069] 本发明的第四方面的方法还可以包括将本发明的第三方面(及在本说明书中描述的其任意实施方案)描述的宿主细胞在适当的条件下在培养基中培养足够时间以获得融合蛋白的表达。

[0070] 培养宿主细胞和分离重组蛋白的方法是本领域中公知的。合适的纯化技术的实例在伴随的实施例中描述。如上文描述的,融合蛋白可以包含亲和标签以帮助使用亲和试剂的纯化,如本领域技术人员已知的。

[0071] 根据本发明的具有增加的生物活性的重组毒素蛋白可以通过公知的方法从重组细胞培养物回收和纯化,所述方法包括硫酸铵或乙醇沉淀、酸提取、阴离子或阳离子交换层析、磷酸纤维素层析,疏水相互作用层析,亲和层析,羟基磷灰石层析和凝集素层析。

[0072] 备选地,根据本发明的具有增加的生物活性的重组毒素蛋白可以从上清回收。在该情况下,通过如本领域技术人员将理解的简单离心将宿主细胞从上清移出。可以使用本领域中已知的标准技术如上述技术从培养基中分离蛋白。

[0073] 本发明人已经确定,根据本发明的具有增加的生物活性的重组毒素蛋白可以用作杀虫剂。

[0074] 本发明的第六方面提供杀虫剂组合物，其包含根据本发明的第一或第四方面及在本说明书中描述的其任意实施方案制备的毒素蛋白。

[0075] 杀虫剂可以是化学物质、生物剂(如病毒或细菌)、抗微生物剂、消毒剂或用于针对任意有害生物的装置。有害生物包括昆虫、植物病原体、杂草、软体动物、鸟、哺乳动物、鱼、线虫类(蛔虫)和破坏财产、传播疾病的微生物或是疾病的载体或引起麻烦。然而，对于本发明，通过“杀虫剂”，我们的意思是，有害生物是破坏财产，特别是农业商品的任何无脊椎动物。

[0076] 更优选地，毒素蛋白能够消灭或至少削弱来自以下目的昆虫有害生物：鞘翅目(Coleopterans)例如黄瓜十一星叶甲(Diabrotica undecimpunctata)；四纹豆象(Callosobruchus maculatus)；鳞翅目(Lepidopterans)例如欧洲玉米螟(Ostinia nubilalis)；烟草天蛾(Manduca sexta)；斑禾草螟(Chilo partellus)；同翅目(Homopteran)有害生物例如褐飞虱(Nilaparvata lugens)；黑尾叶蝉(Nephotettix cinciteps)；小绿叶蝉(Empoasca fabae)；桃蚜(Myzus persicae)；豌豆蚜(Acyrthosiphon pisum)；双翅目(Dipteran)例如麦秆蝇(Chlorop pumilionis)；直翅目(Orthoptera)例如蟋蟀和蝗虫；等翅目(Isoptera)例如白蚁；缨翅目(Thysanoptera)例如牧草虫(thrips)；膜翅目(Hymenoptera)例如蚂蚁和螨目(Acarina)的节肢动物有害生物(螨)。

[0077] 特别优选的有害生物包括鳞翅目甘蓝夜蛾(Mamestra brassicae)、马铃薯叶甲(Leptinotarsa decemlineata，鞘翅目)、麦地种蝇(Delia coarctata，花蝇科(Anthomyiidae))和麦长管蚜(Sitobion avenae，同翅目)。

[0078] 本发明人还研究了根据本发明的方法产生的具有增加的生物活性的重组毒素蛋白是否对软体动物具有杀虫活性。如在伴随的实施例中表明的，他们出人意料地发现，麦蛞蝓(grey field slug)(Decoceras reticulatum，一种软体动物)对根据本发明生产的重组毒素蛋白的杀虫活性易感。因此，根据本发明的重组毒素蛋白能够消灭或至少削弱包括蛞蝓和蜗牛的软体动物，并且特别是麦蛞蝓。

[0079] 优选根据本发明的杀虫组合物是任何所需制剂的形式，如溶液、乳液、喷剂、混悬剂、粉剂、泡沫、糊剂、粒剂、气溶胶、胶囊或其他精细或粗略分开的材料或用于天然或合成材料的浸渍剂。

[0080] 在优选的实施方案，杀虫组合物是与合适的稀释剂、佐剂、防腐剂、分散剂、溶剂、乳化剂等混合的喷剂、混悬剂等的形式。合适的组合物成分是本领域中常规使用的那些，并且尤其是适于本发明口服施用应用的那些。所述组合物可以使用任何合适的溶剂(优选水、醇、矿物油等)、任何合适的固体载体如高岭土、粘土、滑石、白垩、石英、凹凸棒石(attapulgite)、蒙脱石、硅藻土、硅石等，使用作为颗粒支持物的任何固体载体如方解石、大理石、浮石和粉碎的天然纤维材料等来获得。

[0081] 用于本发明的组合物可以另外用于与其他已知的杀昆虫剂、生长促进或调解物质、除草剂、杀真菌剂、增效剂等一起的紧密或物理混合物。

[0082] 所述组合物优选适于与植物或其生长处物理或化学相关，并且适于被病原体口服摄取。

[0083] 所述组合物可以因此包含以重量计0.001％至99％，优选以重量计0.5％至98％，更优选以重量计1.0％至95％的量的融合蛋白(毒素)。

[0084] 上文使用的术语“生长处”是指作物或植物生长的物理位置。例如，对于农作物，生长处可以是田地；对于蔬菜作物，生长处可以是花圃或菜园；对于观赏植物，生长处可以是花盆或容器。

[0085] 本发明的第七方面提供制备根据本发明的第六方面(和本说明书中描述的其任意实施方案)的杀虫剂组合物的方法，所述方法包括将一定量的根据本发明(和本说明书中描述的其任意实施方案)生产的具有增加的生物活性的毒素蛋白与一种或多种合适的载体、稀释剂、佐剂、防腐剂、分散剂、溶剂、乳化剂以有效的杀虫量混合。

[0086] 本发明的第八方面提供预防或治疗植物的有害生物感染的方法，所述方法包括向植物或其生长处施用一定量的根据本发明生产的具有增加的生物活性的毒素蛋白或根据本发明第六方面的杀虫剂组合物(和本说明书中描述的其任意实施方案)；或向植物引入根据本发明的核酸构建体。

[0087] 使用本发明第八方面的方法可以防治众多不同的软体动物有害生物，特别是麦蛞蝓(Decoceras reticulatum)。因此，本发明第八方面的方法包括其中软体动物是蛞蝓或蜗牛，并且特别是麦蛞蝓。

[0088] 本发明的第九方面提供预防或治疗植物的软体动物有害生物感染的方法，所述方法包括向植物或其生长处施用一定量的根据本发明生产的具有增加的生物活性的毒素蛋白或根据本发明第六方面的杀虫剂组合物(和本说明书中描述的其任意实施方案)，或向植物引入根据本发明的核酸构建体。

[0089] 根据本发明和上述的本发明的相关方面和实施方案生产的具有增加的生物活性的毒素蛋白，尤其是杀虫剂组合物，可以用作软体动物杀灭剂。

[0090] 将理解，适当制备和配制所述软体动物杀灭剂，以允许容易被消费者使用。例如，可以将软体动物杀灭剂制备为可以喷在作物上的液体，或也可以施用于作物和/或生长处的颗粒。

[0091] 本领域中公知，软体动物杀灭剂通常以饵料(或丸剂)形式存在。当以这样的形式存在时，使用者可以容易地将软体动物杀灭剂用于植物或其生长处，从而预防或治疗软体动物有害生物感染。

[0092] 因此本发明的第十方面提供杀软体动物诱饵组合物，其包含根据本发明生产的具有增加的生物活性的毒素蛋白和/或根据本发明第六方面的杀虫剂组合物。

[0093] 所述丸剂或饵料还可以包括软体动物引诱剂，从而促进有害生物暴露于软体动物杀灭剂。软体动物引诱剂是引诱软体动物的任何物质。引诱剂可以是助食素。助食素通常用于蛞蝓和蜗牛饵料制剂，以吸引腹足动物摄取软体动物杀灭剂，并且通常是引诱剂和/或食物。还可以使用助食素与其他合适的有机和/或无机载体的混合物。用于软体动物杀灭剂的合适的助食素包括研磨的谷物(如小麦粉、大麦粉、黑麦粉和水稻淀粉)、粉碎的大豆、鱼粉、糖蜜、粉碎的菜籽等。助食素的混合物也可以用于本发明。其他已知引诱剂包括啤酒、酵母和死蛞蝓提取物。饵料组合物还可以包含一种或多种驱鸟剂，如蒽醌。

[0094] 可以配制组合物以提供软体动物杀灭剂随时间的缓慢或延迟释放，从而提供针对软体动物的长期保护。可以用于所述制剂的合适的缓释辅料包括，例如，树脂(如脲/甲醛树脂)、大豆粉、蜡、硬脂酸酯和油(如蓖麻油)。

[0095] 可以用于本发明的饵料或丸剂组合物的其他辅料包括，例如，粘合剂(如甲基纤维

素、聚乙烯吡咯烷酮、聚乙烯醇、聚丙烯酸酯、聚甲基丙烯酸酯、天然蜡、化学改性蜡和合成蜡、糖、淀粉、藻酸盐、琼脂、木质素磺酸酯和阿拉伯胶)、保湿剂(如多元醇,例如糖或甘油)、防腐剂、着色剂和温血物种驱逐剂。

[0096] 还可以将饵料组合物涂层以保护它免于水分降解。这样的涂层可以延长饵料组合物的寿命,并且减少所需的再施用频率。适当地,当施用于潮湿的土壤时,饵料组合物不过早降解。

[0097] 饵料组合物通常以粒剂或丸剂的形式提供。丸剂的大小使得其可以容易地被目标腹足动物消耗以保证摄取。通常,丸剂长约1mm至约5mm。

[0098] 本发明的第十一方面提供转基因植物或其后代,其包含能够表达根据本发明的毒素的根据本发明的核酸构建体。

[0099] "转基因植物"包括的是,植物可以具有结合入其生殖系的根据本发明的核酸构建体或植物可以含有根据本发明的外源核酸构建体,其均可以在植物中表达。

[0100] 将理解,含有根据本发明的核酸构建体的转基因植物,当被以正确方式调节时,将产生根据本发明的方法的具有增加的生物活性的毒素蛋白。产生的蛋白/融合蛋白将作为杀虫剂行使功能。

[0101] 可以将多种不同的植物物种修饰成包括根据本发明的核酸构建体。

[0102] 本领域技术人员将知晓,可以使用任何单子叶或双子叶植物。双子叶植物可以选自包括但不限于以下各项的科:菊科(Asteraceae)、十字花科(Brassicaceae)(例如欧洲油菜(Brassica napus))、藜科(Chenopodiaceae)、葫芦科(Cucurbitaceae)、豆科(Leguminosae)(苏木科(Caesalpiniaceae)、苏木亚科(Aesalpiniaceae)含羞草科(Mimosaceae)、蝶形花科(Papilionaceae)或豆科(Fabaceae))、锦葵科(Malvaceae)、蔷薇科(Rosaceae)或茄科(Solanaceae)。例如,植物可以选自莴苣、向日葵、拟南芥、花椰菜、菠菜、西瓜、南瓜、卷心菜、番茄、马铃薯、青椒、烟草、棉花、秋葵、苹果、玫瑰、草莓、苜蓿、豆、大豆、菜豆、豌豆、小扁豆、花生、鹰嘴豆、杏仁、梨、桃、葡萄藤或柑橘类物种。

[0103] 还包括的是生物燃料和生物能源作物如甘蔗、油菜/加拿大油菜、亚麻籽、柳树、杨树、杂交杨树、柳枝稷、芒草或裸子植物,如火炬松。还包括的是用于青贮饲料(例如饲草玉米)、放牧或草料(例如草、三叶草、sanfoin、苜蓿)、纤维(例如棉花、亚麻)、建筑材料(例如松树、橡树)、纸浆(例如杨树)、化学工业的进料储存(例如高芥酸油菜、亚麻籽)的作物。

[0104] 单子叶植物可以,例如,选自棕榈科(Arecaceae)、石蒜科(Amaryllidaceae)或禾本科(Poaceae)。例如,植物可以是谷类作物,如小麦、水稻、大麦、玉米、燕麦、高粱、黑麦、洋葱、韭菜、粟、荞麦、草坪草、多花黑麦草(Italian rye grass)、柳枝稷、芒草、甘蔗或羊茅属物种。

[0105] 优选,植物是作物植物。作物植物意为以商业规模种植的用于人或动物消费或使用或用于其他非食物/饲料用途的任何植物。优选的植物是玉米、烟草、小麦、水稻、油菜、高粱、大豆、马铃薯、番茄、大麦、豌豆、豆、菜豆、棉花、莴苣、花椰菜或其他芸苔类蔬菜。

[0106] 核酸构建体可以作为转基因引入植物。这可以通过植物遗传改造领域中已知的多种方法进行,例如利用农杆菌、粒子轰击、电穿孔或病毒转化来转化。这样的技术是本领域中公知的。本文中列出的用于各个植物物种的具体技术的使用也是公知的。技术方法可以容易地被技术采用以制备包含根据本发明的核酸序列的转基因植物或其后代。

[0107] 当用于制备本发明的该方面的转基因植物时，核酸构建体通常置于适于在植物中表达核酸序列的“表达盒”内。表达盒将通常含有用于调节核酸在植物中的表达的核酸序列，例如启动子区。

[0108] 一些启动子可以驱动核酸在植物中的组成型表达，包括公知的35S启动子、19S启动子或泛素启动子。

[0109] 其他启动子可以用于调节核酸构建体的器官或组织特异性表达。"组织特异性启动子"或"组织优选启动子"是指并不在所有植物细胞中表达，而仅在特定器官(如叶或种子)、特定组织(如胚或子叶)中的一种或多种细胞类型或特定细胞类型(如叶实质或种子储存细胞)中表达的调节的启动子。这些还包括时间调节的启动子，如在早期或晚期胚发生中，发育中的种子或果实中果实成熟期间，完全分化的叶中，或在顺序的开始。合适的启动子包括来自油菜籽的napin-基因启动子，来自蚕豆(Vicia faba)的USP-启动子，来自拟南芥(Arabidopsis)的油质蛋白(oleosin)-启动子，来自菜豆(Phaseolus vulgaris)的菜豆蛋白(phaseolin)-启动子，来自芸苔(Brassica)的Bce4-启动子或豆球蛋白(legumin)B4启动子，以及在单子叶植物中赋予种子特异性表达的启动子。

[0110] 在光合作用组织中有活性从而驱动绿色组织如叶和茎中的转录的启动子包括核酮糖-1,5-二磷酸羧化酶(RbcS)启动子如来自美洲落叶松(Larix laricina)的RbcS启动子、松树cab6启动子、来自小麦的Cab-1基因启动子、来自菠菜的CAB-1启动子、来自水稻的cablR启动子、来自玉米的丙酮酸正磷酸二激酶(PPDK)启动子、拟南芥SUC2蔗糖-H+共输送体启动子。这样的启动子的代表性核酸序列可以容易地由本领域技术人员自例如GenBank鉴别。

[0111] 适于植物根组织中优先表达的启动子包括例如源自玉米烟酰胺合成酶基因的启动子和水稻RCC3启动子。

[0112] 适于优先在植物脉管组织中表达的启动子包括例如水稻蔗糖合成酶-1基因(RSs1)。

[0113] 诱导型启动子包括响应非生物和生物环境刺激的启动子。非生物环境刺激包括光、温度和水利用度。生物环境刺激包括病原体(包括病毒诱导的、细菌诱导的、真菌诱导的、昆虫诱导的和线虫诱导的启动子)，与共生体和食草动物的相互作用。启动子还可以响应于运动、接触、组织损伤和植物激素(包括脱落酸、细胞分裂素、茁长素、赤霉素、乙烯、油菜素甾醇和肽如系统素(systemin)和结瘤因子(nodulation factor))。时间调节的启动子包括昼夜节律调节的启动子以及相应于非节律时间保持机制的那些。

[0114] 如果希望基因表达以时间特异的方式发生，化学诱导的启动子是特别合适的。这样的启动子的实例是水杨酸诱导型启动子、四环素诱导型启动子和乙醇诱导型启动子(WO 93/21334)。

[0115] 为了在植物中获得改善的表达，可以根据技术改变核酸构建体的密码子使用以形成相当的、改进的或人工的基因或基因部分，从而增加毒素蛋白在植物细胞中的表达效率。此外还可以将核酸插入植物的质体(例如叶绿体)或线粒体基因组中，并使用合适的启动子在那里表达。为了在单子叶植物如玉米或水稻中获得增强的表达，可以将内含子(例如单子叶内含子)添加于嵌合基因。

[0116] 可以优选的是，本发明的(和适于用于本发明的方法的)嵌合核酸还包含用于产物

表达的核酸序列，其可以有助于鉴别其中成功地结合有所述嵌合核酸序列的植物细胞。可以以此方式使用的合适的进一步的核酸序列的实例对于本领域技术人员将是显而易见的，并且包括产生赋予对可以用于选择的物质(如抗生素)的抗性的产物的核酸或产生可以用作选择基础的可检测产物(如显色酶产物)的标志物。

[0117] 本发明的进一步方面提供转化有根据本发明的核酸构建体的植物。

[0118] 本发明的进一步方面提供包含根据本发明的核酸构建体的植物种子。

[0119] 本发明的进一步方面提供根据本发明的核酸构建体或根据本发明制备的毒素蛋白在制造杀虫剂或转基因植物细胞或植物中的用途。

[0120] 本发明的进一步方面提供根据本发明第六方面的杀虫剂(和本说明书中描述的其任意实施方案)消灭或削弱一种或多种有害生物的用途。

[0121] 本发明的进一步方面提供基本上如本文说明书或序列中描述或附图中显示的核酸构建体、毒素蛋白、组合物、载体、宿主细胞、转基因植物、或其制备方法或用途。

[0122] 详细描述

[0123] 现在将参考以下非限制性实施例和附图描述本发明，所述附图显示:

[0124] 图1:含有前区域的毒素的基因结构的示意图

[0125] (A)含有前区域的毒素蛋白的基因结构的示意图。(B)蜘蛛毒素Hv1a 的序列。加框的氨基酸序列对应于前区域。

[0126] 图2:重组Hv1a和pro-Hv1a毒素的表达和纯化

[0127] (A)SDS-PAGE凝胶(20％丙烯酰胺)分析，其显示从培养物上清纯化重组的带Strep标签的Hv1a毒素。泳道1&2是GNA标准物(分别为0.5和0.25μg)并且泳道3&4是利用2.5mM脱硫生物素从Streptactin柱洗脱后的峰级分(10μl)。(B)重组pro-Hv1a的Tris-Tricine凝胶(15％丙烯酰胺)分析，泳道1&2是利用0.2M咪唑从镍亲和柱洗脱后的峰级分(10μl)。箭头指示已经切除组氨酸标签的预测为pro-Hv1a的主要蛋白产物。(C)使用抗-His抗体对(B)中样品的蛋白质印迹分析。

[0128] 图3:纯化的pro-Hv1a/GNA、Hv1a/GNA和MODHv1a/GNA的冻干的样品的SDS-PAGE凝胶分析

[0129] 纯化的重组的含有Hv1a的融合蛋白的SDS-PAGE分析(17.5％丙烯酰胺凝胶)，用考马斯蓝进行针对总蛋白的凝胶染色。如下上样:泳道1:pro-Hv1a/GNA;泳道2:Hv1a/GNA;泳道3:MODHv1a/GNA;泳道4-6:分别为1、2和4μg的GNA标准物;泳道7-9:12.5、25和50μg冻干的纯化的pro-Hv1a/GNA(以使得能够定量融合蛋白含量)。

[0130] 图4:重组pro-Hv1a、pro-Hv1a/GNA和Hv1a/GNA对甘蓝夜蛾(Mamestra brassicae)的注射毒性

[0131] 注射不同剂量的重组pro-Hv1a、pro-Hv1a/GNA或Hv1a/GNA后第3-5龄甘蓝夜蛾幼虫的存活百分数。(A)注射各种剂量的pro-Hva1后第5龄幼虫的存活百分数。(B)注射各种剂量的pro-Hv1a/GNA(剂量A)或Hv1a/GNA(剂量B)后第3-4龄幼虫的存活百分数。(C)注射各种剂量的pro-Hv1a/GNA(剂量A)和Hv1a/GNA(剂量B)后第5龄幼虫的存活百分数。

[0132] 图5:重组pro-Hv1a、Hv1a/GNA、pro-Hv1a/GNA和GNA对甘蓝夜蛾的摄取毒性

[0133] 摄取单次2μl含有20μg纯化的pro-Hv1a、Hv1a/GNA、pro-Hv1a/GNA或GNA的小滴后第3龄甘蓝夜蛾幼虫的存活百分数。对照幼虫以不含有添加的蛋白的小滴喂食(每次处理n

＝10)。

[0134] 图6:重组pro-Hv1a、pro-Hv1a/GNA、Hv1a/GNA或GNA对豌豆蚜(Acyrthosiphon pisum)和麦长管蚜(Sitobion avenae)的摄取毒性

[0135] 在含有0.05-0.75mg/ml纯化的重组pro-Hv1a、pro-Hv1a/GNA、Hv1a/GNA或GNA的人工饲料的情况下的(A)豌豆蚜(豌豆蚜虫)和(B)麦长管蚜(麦蚜)的存活百分数。

[0136] 图7:重组pro-Hv1a/GNA和Hv1a/GNA对麦蛞蝓(Deroceras reticulatum)的注射毒性

[0137] 被注射以100、50或25μg的Hv1a/GNA或pro-Hv1a/GNA的麦蛞蝓(200mg±40mg)的存活百分数。对于对照处理,n＝18;对于100μg剂量,n＝10;并且对于50和25μg剂量,n＝8。

[0138] 图8:重组PI1a和Ao1bPro-PI1a毒素的表达和纯化

[0139] (A)在“普通”SDS-PAGE上分离的源自编码成熟毒素序列的构建体的PI1a毒素;M表示标志物,PI1a的上样是5和10μg。(B)用6M脲变性后在SDS-PAGE上分离的PI1a毒素(5μg)。(C)在SDS-PAGE上指定为Ao1b的源自含有前区域的构建体的重组PI1a毒素,Ao1bPro-PI1a的上样是2.5μg。(D)使用抗-His抗体对纯化的Ao1bPro-PI1a(25、50&100ng)的蛋白质印迹。

[0140] 图9:通过SDS-PAGE表征纯化的重组PI1a/GNA融合蛋白

[0141] (A)PI1a/GNA融合蛋白(10μg)和GNA标准物(5μg)的SDS-PAGE分析。(B)使用PNGase F的PI1a/GNA融合蛋白的去糖基化(空心箭头指示的条带),GNA标准物(5μg)。(C)Ao1bPro-PI1a/GNA(1、2和4μg)的SDS-PAGE分析,GNA标准物(5μg)。(D)Hv1aPro-PI1a/GNA(1、2和4μg)的SDS-PAGE分析,GNA标准物(1、2和4μg)。

[0142] 图10:重组PI1a和Ao1bPro-PI1a对甘蓝夜蛾的注射毒性

[0143] (A)注射不同剂量的纯化的重组PI1a后第5龄甘蓝夜蛾幼虫的存活百分数。(B)注射不同剂量的纯化的重组PI1a(剂量A)或Ao1bPro-PI1a(剂量B)后第5龄甘蓝夜蛾幼虫的存活百分数。

[0144] 图11:重组PI1a/GNA、Ao1bPro-PI1a/GNA和Pro-HV1a-PI1a/GNA对甘蓝夜蛾的注射毒性

[0145] 注射不同剂量的纯化的重组PI1a/GNA(A)、Ao1b-ProPI1a/GNA(B)或Pro-Hv1a-PI1a/GNA(C)后第5龄甘蓝夜蛾幼虫的存活百分数。每次处理n＝20。

[0146] 图12:重组PI1a/GNA、Ao1bPro-PI1a/GNA和Hv1aPro-PI1a/GNA对甘蓝夜蛾的摄取毒性

[0147] 摄取单次2μl含有20μg纯化的PI1a/GNA(A)、Ao1bPro-PI1a/GNA(B)或Hv1aPro-PI1a/GNA(C)融合蛋白的小滴后第3龄甘蓝夜蛾幼虫的存活百分数。在所有情况中,对照是单独的蔗糖(无添加的蛋白);30μg的任一种PI1a毒素(成熟或修饰形式)或GNA。

[0148] 缩写

[0149] BB:结合缓冲液

[0150] ECL:增强的化学发光

[0151] HRP:辣根过氧化物酶

[0152] PBS:磷酸盐缓冲盐水

[0153] SDS-PAGE:十二烷基硫酸钠聚丙烯酰胺凝胶电泳

[0154] YPG:酵母提取物蛋白胨甘油

[0155] 材料和方法

[0156] 重组Hv1a、pro-Hv1a和pro-Hv1a/GNA融合蛋白的克隆

[0157] 使用一系列重叠的寡核苷酸组装编码成熟Hv1a氨基酸序列的合成的基因，针对在酵母中的表达优化密码子选择(Fitches等人，2012)。为了产生编码成熟Hv1a肽的表达构建体，编码序列通过PCR使用具有PstI和SalI位点的引物扩增并按照制造商的方案中描述的使用QiaQuick凝胶提取试剂盒(Qiagen)从切下的凝胶条纯化。将提取的DNA片段消化(PstI和SalI)并连接到同样消化的酵母表达载体pGAPZαB(Invitrogen)中，所述载体在之前已被修饰成含有与酵母α-因子前体前序列(pre-pro-sequence)同框的5’Strep标签。将得到的质粒转化入电感受态大肠杆菌细胞并且通过凝胶电泳和DNA测序检查选择的克隆的构建体的正确组装。

[0158] 将pro-Hv1a编码序列通过PCR使用具有PstI和XbaI位点的引物(正向：TACTGCAGCAGAAGATACTAGAGCT和反向:ATTCTAGAATCACATCTCTTAAC)扩增。将凝胶提取的产物用PstI和XbaI消化并连接到同样消化的酵母表达载体pGAPZαB中。将得到的重组质粒转化入大肠杆菌细胞并且通过凝胶电泳和DNA测序检查选择的克隆的构建体的正确组装。

[0159] 为了产生pro-Hv1a/GNA构建体，将pro-Hv1a编码序列通过PCR使用具有PstI和NotI位点的引物(正向：TACTGCAGCAGAAGATACTAGAGCT和反向：ATGCGGCCGCATCACATCTCTTAAC)扩增并如上所述通过凝胶电泳纯化。用PstI和NotI限制性酶切后，将PCR产物连接入之前产生的用相同酶消化的含有成熟GNA编码序列的pGAPZαB质粒中。含有编码pro-Hv1a/GNA融合蛋白的表达载体的选择的克隆通过DNA测序验证。

[0160] Hv1a构建体的序列显示如下：

[0161] 天然Hv1a:SPTCIPSGQPCPYNENCCSQSCTFKENENGNTVKRCD

[0162] 重组Hv1a(α因子信号序列,Hv1a毒素,Strep标签绿色突出显示区域,无前区域,无载体)：

[0163] MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEAAWSHPQFEKGLQSPTCIPSGQPCPYNENCCSQSCTFKENENGNTVKRCD

[0164] 重组pro-Hv1a(α因子信号序列,Hv1a毒素,前区域,无载体,$(His)_6$标签)：

[0165] MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEAAAEDTRADLQGGEAAEKVFRRSPTCIPSGQPCPYNENCCSQSCTFKENENGNTVKRCDALEQKLISEEDLNSAVDHHHHHH

[0166] 重组Hv1a/GNA(α因子信号序列,Hv1a毒素,无前区域,载体)：

[0167] MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEAAASPTCIPSGQPCPYNENCCSQSCTFKENENGNTVKRCDAAADNILYSGETLSTGEFLNYGSFVFIMQEDCNLVLYDVDKPIWATNTGGLSRSCFLSMQTDGNLVVYNPSNKPIWASNTGGQNGNYVCILQKDRNVVIYGTDRWATGVD

[0168] 重组pro-Hv1a/GNA(α因子信号序列,Hv1a毒素,前区域,载体,$(His)_6$标签)：

[0169] MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEAAAEDTRADLQGGEAAEKVFRRSPTCIPSGQPCPYNENCCSQSCTFKENENGNTVKRCDAAADNILYSGETLSTGEFLNYGSFVFIMQEDCNLVLYDVDKPIWATNTGGLSRSCFLSMQTDGNLVVYNPSNKPIWASNTGGQNGNYVCILQKDRNVVIYGTDRWATGVDHHHHHH

[0170] 重组PI1a和PI1a/GNA融合蛋白的克隆

[0171] 结合编码成熟PI1a毒素(P83256)的序列的双链DNA(针对酵母优化密码子选择)由本发明人设计并由ShineGene Molecular Biotech,Inc.(Shanghai 201109,China;http://www.synthesisgene.com/)合成和提供在载体pUC57中。其他寡核苷酸由Sigma Chemical Co提供。

[0172] 通过用PstI和XbaI消化,通过琼脂糖凝胶电泳分离编码序列片段,接着连接于用相同酶消化的pGAPZαB,来将Pl1a编码序列从pUC57转移到酵母表达载体pGAPZαB(Invitrogen)。使用QiaQuick凝胶提取试剂盒(Qiagen)从切下的凝胶条纯化DNA片段。使用标准方案通过转化电感受态大肠杆菌细胞将得到的重组质粒克隆。通过DNA测序检查选择的克隆的构建体的正确组装。为了产生修饰的构建体以表达Pl1a,将编码来自U3-agatoxin-Ao1b(Q5Y4V7)的前区域的两条互补的合成的寡核苷酸组装并插入Pl1a表达构建体的PstI位点。构建体(ProAo1b-PI1a)的正确组装通过DNA测序检查。

[0173] 为了产生编码Pl1a/GNA融合蛋白的构建体,将来自pGAPZαB中验证的表达构建体的成熟PI1a编码序列通过用PstI和NotI消化切下并通过琼脂糖凝胶电泳纯化。将含有融合蛋白构建体Hv1a/GNA的pGAPZαB质粒(Fitches等人,2012)用PstI和NotI消化以去除Hv1a编码序列并通过琼脂糖凝胶电泳纯化。随后通过连接纯化的片段并克隆得到的重组质粒,将Hv1a编码区用Pl1a替代。为了产生含有来自U3-agatoxin-Ao1b的前区域的Pl1a/GNA的修饰的表达构建体,如上所述修饰Pl1a/GNA表达构建体;此外,还将来自pro-Hv1a毒素的前区域用于被称为Pro-Hv1a-PI1a的另一个构建体。通过DNA测序验证含有编码PI1a/GNA融合蛋白的表达载体的选择的克隆。所有DNA测序使用Applied Biosystems ABI Prism 3730自动DNA测序仪,由DBS Genomics,School of Biological and Biomedical Sciences,Durham Universitv,UK进行。

[0174] PI1a构建体的序列如下所示:

[0175] 天然PI1a:GCLGEGEKCADWSGPSCCDGFYCSCRSMPYCRCRNNS

[0176] 重组PI1a(α因子信号序列,PI1a毒素,无前区域,无载体,$(His)_6$标签):

[0177] MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEAAAGCLGEGEKCADWSGPSCCDGFYCSCRSMPYCRCRNNSALEQKLISEEDLNSAVDHHHHHH

[0178] 重组Ao1bPro-PI1a(α因子信号序列,前区域[Ao1b],PI1a毒素,无载体,+$(His)_6$标签):

[0179] MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEAAAISYEEGKELFQKERGCLGEGEKCADWSGPSCCDGYCSCRSMPYCRCRNNSALEQKLISEEDLNSAVDHHHHHH

[0180] 重组PI1a/GNA(α因子信号序列,PI1a毒素,无前区域,载体,$(His)_6$标签):

[0181]
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEAAAGCLGEGEKCADWSGPSCCDGFYCSCRSMPYCRCRNNSAAADNILYSGETLSTGEFLNYGSFVFIMQEDCNLVLYDVDKPIWATNTGGLSRSCFLSMQTDGNLVVYNPSNKPlWASNTGGQNGNYVCILQKDRNVVIYGTDRWATGVDHHHHHH

[0182] 重组Aolb Pro-PI1a/GNA(α因子信号序列,前区域[Aolb],PI1a毒素,载体,$(His)_6$标签):

[0183] MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEAAAISYEEGKELFQKERGCLGEGEKCADWSGPSCCDGCSCRSMPYCRCRNNSAAADNILYSGETLSTGEFLNYGSFVFIMQEDCNLVLYDVDKPIWATNTGGLSRSCFLSMQTDGNLVVYNPSNKPIWASNTGGQNGNYVCILQKDRNVVIYGTDRWATGVDHHHHHH

[0184] 重组Hv1a Pro-PI1a/GNA(α因子信号序列,前区域[Hv1a],PI1a毒素,载体,$(His)_6$标签):

[0185] MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEAAAEDTRADLQGGEAAEKVFRRGCLGEGEKCADWSGPCCDGFYCSCRSMPYCRCRNNSAAADNILYSGETLSTGEFLNYGSFVFIMQEDCNLVLYDVDKPIWATNTGGLSRSCFLSMQTDGNLVVYNPSNKPIWASNTGGQNGNYVCILQKDRNVVIYGTDRWATGVDHHHHHH

[0186] Hv1a、pro-Hv1a、Hv1a/GNA和pro-Hv1a/GNA融合蛋白在酵母中的表达

[0187] 将含有Hv1a、pro-Hv1a、Hv1a/GNA和pro-Hv1a/GNA序列的pGAPZαB质粒在大肠杆菌中扩增,纯化并用BlnI(Takara)线性化。将线性化的质粒使用EasyComp转化试剂盒(Invitrogen)如制造商的方案中所述转化入巴斯德毕赤酵母株SMD1168H(Invitrogen)。在含有博莱霉素(100mg/ml)的YPG琼脂板(1%酵母提取物(w/v),2%蛋白胨(w/v),4%甘油(v/v),1.5%琼脂(w/v))上选择转化的酵母克隆。通过分析来自在YPG-博莱霉素培养基中在30℃生长2-3天的小规模摇瓶培养物(10ml)的培养物上清检查选择的克隆的表达。通过SDS-聚丙烯酰胺凝胶电泳分离上清样品;将凝胶印迹在硝酸纤维素上并用抗-$(His)_6$一抗(BioRad)或抗-Strep抗体探测,或对于Hv1a/GNA和pro-Hv1a/GNA印迹,用抗-GNA一抗探测,接着洗涤,用HRP-缀合的二抗(BioRad)探测,并通过ECL检测结合的抗体,如前所述(Fitches等人,2001;2012)。

[0188] 为了蛋白生产,选择的含有整合的Hv1a、pro-Hv1a、Hv1a/GNA和pro-Hv1a/GNA表达盒的巴斯德毕赤酵母克隆在7.5L BioFlo 110台式发酵罐(New Brunswick Scientific)或5L Bio-Controlly ADI1010台式发酵罐(APPLIKON BIOTECHNOLOGY,Holland)中生长。将转化的巴斯德毕赤酵母的YPG培养物(200ml)在30℃震荡培养2-3天(无博莱霉素抗生素),之后接种2.5L补充有PTM1盐的无菌最小培养基。用4天时间,在增加的甘油进料(5-10ml/h;总共1.25l)的情况下,进行在30℃,30%溶解氧,pH 4.5-5.0,在连续搅拌下的培养。随后通过离心(20min,8000rpm;4℃)将培养物上清与细胞分离,经2.7μM和0.7μM玻璃纤维滤器(GFD和GFF;Whatmann)过滤来澄清。仅对于Hv1a,通过加入4x浓缩储液将上清调节至含有0.3M氯化钠,pH 8.0的50mM磷酸盐缓冲液。在streptactin柱(1 ml)上,以0.5ml/min的流速纯化重组Hv1a。在含有0.3M氯化钠的pH 8.0的50mM磷酸盐缓冲液中平衡柱。使用2.5mM脱硫生物素(在磷酸盐缓冲液中;pH 8.0)从柱上洗脱带Strep标签的Hv1a。对于所有其他蛋白,通过加入4x浓缩储液(4X结合缓冲液[BB])将上清调节至0.02M磷酸钠缓冲液,0.4M氯化钠,pH 7.4。通过镍亲和层析在5ml HisTrap粗制镍柱(GE Healthcare)上以2ml/min的流速纯化重组pro-Hv1a Hv1a/GNA和pro-Hv1a/GNA。上样后,将柱用1xBB(50mM磷酸钠;0.4M氯化钠)洗涤并随后用含有0.025M咪唑的BB洗涤,并且最后用含有0.2M咪唑的BB将结合的重组蛋白洗脱。在所有情况下,之后通过SDS-PAGE检查洗脱的蛋白的纯度,使用多次改变对去离子水进

行透析以去除所有小分子，并冷冻干燥。通过与SDS-PAGE凝胶上运行的GNA标准物的已知量相比或通过使用BCA™蛋白测定试剂盒(Thermo Scientific)的BCA分析评估重组蛋白的浓度。

[0189] PI1a和PI1a/GNA融合蛋白在酵母中的表达

[0190] 将含有PI1a和PI1a/GNA表达构建体的pGAPZαB质粒在大肠杆菌中扩增，纯化并用BlnI线性化。使用EasyComp转化试剂盒(Invitrogen)如制造商的方案中所述将线性化的质粒转化入巴斯德毕赤酵母株SMD1168H(Invitrogen)。将转化的酵母克隆在含有博莱霉素(100mg/ml)的YPG琼脂板(1％酵母提取物(w/v)，2％蛋白胨(w/v)，4％甘油(v/v)，1.5％琼脂(w/v))上铺板并选择。通过分析来自在YPG-博莱霉素培养基中在30℃培养2-3天的小规模摇瓶培养物的培养物上清检查选择的克隆(对于每种构建体至少10个)的重组蛋白表达。通过SDS-聚丙烯酰胺凝胶电泳分离上清样品；将凝胶印迹在硝酸纤维素上，并用抗-(His)$_6$一抗(BioRad)或抗-GNA一抗探测，接着洗涤，用HRP-缀合的二抗(BioRad)探测，并通过ECL检测结合的抗体。

[0191] 将选择的含有整合的PI1a和PI1a/GNA构建体的巴斯德毕赤酵母的克隆在5L Bio-Controlly ADI1010台式发酵罐(Applikon Biotechnology，Holland)中培养。对于发酵，将两个含有毒素或融合基因的巴斯德毕赤酵母的100ml YPG培养物在30℃在振荡下培养2-3天，之后将其用于接种2.5L补充有PTM1盐的无菌最小培养基。用4天时间，在增加的甘油进料(5-10ml/h)的情况下，进行在30℃，30％溶解氧，pH 4.5-5.0的在连续搅拌下的培养。通过离心(20min，5000g)将培养物上清与细胞分离，并通过加入5x浓缩储液调节至0.02M磷酸钠缓冲液，0.4M氯化钠，pH 7.4。将重组蛋白通过镍亲和层析在5ml HisTrap粗制镍柱(GE Healthcare)上，以2ml/min的流速纯化。上样后，将柱用0.02M磷酸钠缓冲液，0.4M氯化钠pH 7.4洗涤并将结合的蛋白用在相同缓冲液中的0.2M咪唑洗脱。通过SDS-PAGE检查洗脱的蛋白的纯度，使用多次改变对去离子水进行透析以去除所有小分子，并冷冻干燥。通过与SDS-PAGE凝胶上运行的GNA标准物的已知量相比或通过使用BCA™蛋白测定试剂盒(Thermo Scientific)的BCA分析评估重组蛋白的浓度。

[0192] 昆虫生物测定

[0193] 将第3-5龄甘蓝夜蛾幼虫(重约30-55mg)用于注射生物测定。给幼虫注射在5μl PBS(磷酸缓冲盐水；0.15M NaCl，0.015M磷酸钠缓冲液，pH7.2)中的不同剂量的Hv1a、pro-Hv1a、pro-Hv1a/GNA、Pl1a、Ao1bProPl1a、Pl1a/GNA、Ao1bPro-Pl1a/GNA或Hv1aPro-Pl1a/GNA(每个剂量n＝20)。给对照幼虫注射5μl 1×PBS。注射后12-96h记录瘫痪和死亡率。

[0194] 进行小滴-喂食测定以评价Hv1a/GNA、pro-Hv1a/GNA、Pl1a、Ao1bProPl1a、Pl1a/GNA、Ao1bPro-Pl1a/GNA或Hv1aPro-Pl1a/GNA对甘蓝夜蛾的第三至第五龄幼虫的口服活性。将幼虫饥饿大约24h，之后喂食以促进小滴消耗。用2μl含有在含有10％蔗糖(w/w)的1x PBS溶液中的20μg上述融合蛋白，30μg毒素或30μg GNA的小滴喂食幼虫。用不含有添加蛋白的PBS/蔗糖小滴喂食对照幼虫。消耗小滴后，将处理的幼虫置于标准人工饲料上。

[0195] Hv1a/GNA和pro-Hv1a/GNA对豌豆蚜(豌豆蚜虫)和麦长管蚜(麦蚜)的杀昆虫效应通过喂食100μl含有已知浓度的融合蛋白的液体人工饲料(Prosser和Douglas，1992)，使用双parafilm小袋(饲料小滴在中间)将饲料递送至昆虫来评价。所述实验使用1-2日龄蚜虫并且每日评价存活达六天。

[0196] 注射生物测定:麦蛞蝓(软体动物麦蛞蝓)

[0197] 通过注射到成体蛞蝓(0.2-0.3g)中测试Hv1a/GNA和pro-Hv1a/GNA对成体蛞蝓(麦蛞蝓)的活性。将蛞蝓在4℃冰冷(达约15分钟),之后注射重悬在20μl PBS中的25μg、50μg或100μg的纯化的融合蛋白。每日评价死亡率达7天。

[0198] 统计分析

[0199] 使用Kaplan-Meier存活分析,使用Prism(v5)软件分析存活数据。所有其他数据分析使用Origin 8.5制图和数据分析软件进行。进行ANOVA分析(利用Bonferroni-Dunn事后检验)以确定处理之间在测量的参数方面的任何显著差异。

[0200] 结果

[0201] 本发明人进行实验以研究在用于毒素的表达构建体中包含前区域对所述毒素的生物活性的影响。

[0202] 研究Hv1a的毒性的实验

[0203] 引言

[0204] 为了研究包含前区域对重组毒素的毒性的影响,使用ω-Hexatoxin-Hv1a。ω-Hexatoxin-Hv1a是分离自蓝山漏斗网蜘蛛(Hadroncyhe versuta)的毒素。ω-Hexatoxin-Hv1a(或ω-ACTX-Hv1a)是钙通道拮抗剂,并且之前证明ω-ACTX-Hv1a可以阻断无脊椎动物钙通道但不阻断脊椎动物钙通道。

[0205] 尽管已经表明,在局部用于毛虫时,ω-ACTX-Hv1a本身可以用作杀虫剂(Khan等人,2006),但是没有单独的该肽的杀昆虫活性的进一步证据。在专利申请PCT/GB2012/000287中,本发明人表明巴斯德毕赤酵母中表达的重组毒素(ω-ACTX-Hv1a)的毒性可以通过将该蛋白与植物凝集素GNA融合表达来增强,之前证明所述植物凝集素GNA跨消化道上皮并将'乘客(passenger)'肽从无脊椎动物的消化道递送至循环系统。

[0206] 为了进一步研究体外表达的毒素的效力可能怎样改善,本发明人分析了编码节肢动物毒素的基因的DNA序列。用于PCT/GB2012/000287的节肢动物毒素是小的,富含半胱氨酸的蛋白,其属于多个蛋白序列超家族(其包括来自除节肢动物之外的生物的毒素)。编码基因包括两条最终蛋白产物中不存在的序列;在翻译期间去除的预测的N-端信号肽和信号肽和分离的蛋白最终序列之间的预测的前区域(参见图1A)。

[0207] 本发明人研究在表达构建体中包含前区域对重组蛋白的整体毒性的影响。

[0208] 在第一个例子中,使用ω-ACTX-Hv1a,因为该毒素在其基因序列中含有预测的前区域(参见图1B)。

[0209] 重组Hv1a、pro-Hv1a、Hv1a/GNA和pro-Hv1a/GNA的合成基因构建体、表达和纯化

[0210] 基于载体pGAPZαB合成重组Hv1a、pro-Hv1a、Hv1a/GNA和pro-Hv1a/GNA融合蛋白构建体,所述载体pGAPZαB具有强的组成型启动子(GAPDH)以指导靶基因表达。pGAPZ载体是表达宿主巴斯德毕赤酵母中的整合载体并且选择的转化子含有整合入宿主基因组的表达构建体。用于表达重组Hv1a和pro-Hv1a的构建体含有对应于毒素的公开序列的预测的氨基酸序列。对于Hv1a,将成熟肽克隆在酵母α-因子前体前序列和编码Strep标签的8个氨基酸(即经3氨基酸接头'GLQ'连接于成熟Hv1a序列的N-末端的WSHPQFEK)的框内。对于Pro-Hv1a,N-端前区域排列在编码酵母α-因子前体前序列的序列的C-端框内,并且构建体还含有编码myc表位和$(His)_6$标签的序列(由载体提供,在预测的产物的C-末端)。对于Hv1a/GNA,将成

熟毒素序列经3氨基酸(AAA)接头肽融合于对应于成熟雪花莲凝集素(GNA)的残基1-105的编码序列的N-末端。对于pro-Hv1a/GNA,将合成的pro-Hv1a编码序列经3氨基酸(AAA)接头肽融合至对应于成熟雪花莲凝集素(GNA)的残基1-105的编码序列的N-末端。两种融合蛋白构建体也均排列在酵母α-因子前体前序列,和编码$(His)_6$标签的序列(由载体提供)的C-末端的框内。通过核酸内切酶消化的DNA的连接组装构建体并在克隆后通过DNA测序检查。

[0211] 将序列验证的克隆(含有重组Hv1a、pro-Hv1a、Hv1a/GNA或pro-Hv1a/GNA)转化入巴斯德毕赤酵母蛋白酶缺陷株SMD1168H,并使用抗生素博莱霉素选择。将选择的克隆在在30℃摇瓶培养中生长3-4天并使用蛋白质印迹分析培养物上清中重组蛋白的表达。这使得高表达克隆能够被选择用于通过台式发酵生产。分析的转化的酵母克隆中的大多数显示蛋白表达的证据,如由在蛋白质印迹上具有预期大小的免疫反应性条带的存在判断的(结果未示)。

[0212] 选择的克隆的发酵在控制的环境条件下在生物反应器中进行。使用引导表达蛋白分泌出细胞并进入生长培养基中的pGAPα因子分泌信号,使得能够随后从发酵的培养物上清纯化重组蛋白。通过离心获得上清,通过过滤澄清,并随后将重组蛋白通过亲和层析(对于Hv1a用Streptactin,并且对于pro-Hv1a和pro-Hv1a/GNA用镍亲和)纯化。含有目标蛋白的洗脱峰通过透析脱盐并冻干。对于重组蛋白的产量,以大约5-10mg/L培养物上清产生Hv1a;以大约40mg/L产生pro-Hv1a,如通过BCA定量估计的,并且以大约40mg/L产生Hv1a/GNA,和以大约21mg/L产生pro-Hv1a/GNA,如通过半定量SDS-PAGE估计的。

[0213] 如图2A(泳道3和4)中所示,在SDS-PAGE凝胶分离为大约6.5kDa的单个蛋白的带5'Strep标签的成熟纯化的Hv1a,与5.47kDa的预测的分子量相当(未将凝胶优化以用于分离小分子量蛋白)。使用Tris-Tricine SDS-PAGE凝胶分离纯化的重组pro-Hv1a并且通过对总蛋白染色和使用抗-His抗体的蛋白质印迹来分析(图2B&C)。在Tris-Tricine凝胶上,重组毒素pro-Hv1a产生在大约4kDa的主蛋白条带和进一步的在分子量范围6-16kDa内的较弱条带。大约4kDa的显著条带不与抗-$(His)_6$抗体免疫反应(图2C),并且分子量与切割C-端标签区域后的预测的分子量(4.06kDa)一致。不带His标签的蛋白连同带His标签的蛋白从镍亲和柱的拉下(Pull down)之前对于从毕赤酵母上清回收的其他重组蛋白已经观察到。显示与抗-$(His)_6$抗体的阳性免疫反应性的10kDa蛋白对应于包括前区域的重组Hv1a的预测的分子量(9kDa),提示在酵母细胞的加工期间前区域的切割不完全。考虑到含有C-端His域但无前区域的Hv1a预测的分子量是6.74kDa,也与抗-$(His)_6$抗体免疫反应的14kDa条带可能代表Hv1a的二聚形式。

[0214] 在SDS-PAGE凝胶上分析纯化的pro-Hv1a/GNA、Hv1a/GNA和MODHv1a/GNA的冻干的样品(图3)。所有融合蛋白都观察到大约19kDa和14kDa的两个条带。Hv1a/GNA的预测分子量是16.27kDa,而没有前区域但含有$(His)_6$标签的pro-Hv1a/GNA的预测分子量是16.95,均稍小于观察到的19kDa条带。然而,pro-Hv1a/GNA和Hv1a/GNA在SDS-PAGE凝胶上的相同分离提示,前区域在巴斯德毕赤酵母细胞的加工期间已从pro-Hv1a/GNA切割。完整MODHv1a/GNA的预测分子量是17.09kDa,并且相应地,与Hv1a/GNA和pro-Hv1a/GNA相比,由于存在在Hv1a/GNA中不存在的另外的组氨酸标签,该蛋白作为稍大的蛋白跑胶。在所有情况下,较小的14kDa条带都与GNA抗体免疫反应(结果未示)并且在大小上对应于已经切去Hv1a毒素的GNA。如之前对于Hv1a/GNA所观察到的(Fitches等人,2012),完整pro-Hv1a/GNA融合蛋白与

切割的GNA的比例评估为约1:1，如通过对SDS-PAGE凝胶的考马斯蓝染色判断的，而MODHv1a/GNA中Hv1a序列的修饰导致完整融合蛋白的较高回收(完整:切割比2:1)。Hv1a/GNA融合蛋白的定量是基于与已知浓度的GNA标准物相比的条带强度，如图3中所示。

[0215] 重组Hv1a、pro-Hv1a、Hv1a/GNA和pro-Hv1a/GNA融合蛋白对菜蛾(甘蓝夜蛾)幼虫的注射毒性

[0216] 以多至100μg的重组毒素的剂量注射Hv1a不导致任何幼虫死亡，存活与对照相当(n＝40;存活>90％)。这表明，没有N-端前区域的成熟Hv1a肽的表达导致无生物活性的毒素的产生，提示在由酵母细胞合成期间毒素未正确加工和/或折叠。相反，用pro-Hv1a、Hv1a/GNA、MODHv1a/GNA或pro-Hv1a/GNA注射新羽化的第3-4(～30-40mg)和第5龄(～45-55mg)甘蓝夜蛾幼虫导致显著的幼虫死亡率。

[0217] 如图4A中所示，重组pro-Hv1a在注射到第5龄幼虫后的效应是剂量依赖性的。10μg和20μg pro-Hv1a/昆虫的注射剂量导致注射后24小时完全死亡，并且注射5μg/昆虫导致注射后24小时80％死亡。在1.25μg/昆虫的最低剂量，所有昆虫显示无力的瘫痪和暂时的不进食，尽管一些瘫痪的昆虫在注射后2-3小时恢复并能够继续进食。从这些测定，估计的重组pro-Hv1a的LD_{50}(致死剂量;48小时)是25μg/g昆虫。如表1中总结的，这比之前公布的在大肠杆菌中产生的重组Hv1a毒素的LD_{50}(其LD_{50}(72小时)是～69μg/g昆虫)低约3倍。

[0218] 如图4B&C中所示，与Hv1a/GNA相比，用pro-Hv1a/GNA注射第3-4和第5龄甘蓝夜蛾幼虫显示增加的毒性。例如，与注射相同剂量的pro-Hv1a/GNA后24小时记录的100％死亡相比，向第5龄幼虫注射10μg Hv1a/GNA导致72小时后50％死亡。在2.5μg/昆虫的pro-Hv1a/GNA剂量观察到显著的幼虫死亡率(75％)，而注射5或10μg Hv1a/GNA不导致第5 龄幼虫任何显著的死亡水平。在注射较小的第3-4龄幼虫后观察到相似的结果(图4B)。如表1中所示，与Hv1a/GNA的55μg/g昆虫的LD_{50}相比，估计的pro-Hv1a/GNA的LD_{50}值低约10倍(4.6μg/g昆虫)。这表明，与源自编码融合于GNA的成熟毒素序列的构建体的蛋白相比，将前区域添加至Hv1a/GNA构建体导致显著更具毒性的融合蛋白的产生。更出人意料地，计算的pro-Hv1a/GNA的4.6μg/g昆虫的LD_{50}值比估计的pro-Hv1a的25μg/g昆虫低约5倍。这表明，pro-Hv1a与GNA的连接得到具有比单独的pro-Hv1a或没有前区域的Hv1a/GNA高的生物活性的蛋白。计算的pro-Hv1a/GNA的4.6μg/g昆虫的LD_{50}值也比天然Hv1a的文献值低超过2倍(与重组Hv1a相对;参见表1)。该文献值代表在实夜蛾(Heliothis)(与甘蓝夜蛾相同的昆虫目中的物种)的LD_{50}值。我们猜测，将GNA与pro-Hv1a融合进一步作用以促进巴斯德毕赤酵母细胞对Hv1a毒素的正确加工和折叠，允许要获得的生物活性的进一步增加。以多至40μg/昆虫单独注射GNA不导致甘蓝夜蛾幼虫的死亡。

[0219] 表1:在利用甘蓝夜蛾幼虫的注射生物测定中重组毒素和融合蛋白的毒性

[0220]

	Hv1a (文献)	Hv1a	Pro-Hv1a	Hv1a/GNA	Pro-Hv1a/GNA	Hv1a (*大肠杆菌*)
LD_{50}	12 μg/g (实夜蛾属)*	>1000 μg/g	25 μg/g (48h) < 25 μg/g (72h)	55 μg/g (72h)	4.6 μg/g (48h)	69 μg/g (72h)

[0221] *未获得甘蓝夜蛾的数据

[0222] 重组Hv1a、pro-Hv1a和pro-Hv1a/GNA融合蛋白对菜蛾(甘蓝夜蛾)幼虫的摄取毒性

[0223] 通过将2μl含有20μg融合蛋白的小滴饲喂给羽化的第三龄甘蓝夜蛾幼虫评估proHv1a/GNA和Hv1a/GNA的口服活性。除了未添加蛋白的对照组以外，对照处理是20μg的GNA或pro-Hv1a之一。如图5和表2中所示，仅用pro-Hv1a/GNA饲喂的幼虫观察到显著的效应，在摄取一滴融合蛋白后5天记录到90％死亡率。相反，对于Hv1a/GNA融合蛋白，死亡率仅为30％，仅稍高于对于分别用GNA和pro-Hv1a处理观察到的20％和15％死亡率。在发现在4天时间内20μg pro-Hv1a/GNA融合蛋白的单一剂量导致第五龄幼虫30％死亡率的测定中观察到类似的结果，而对于用20μg的Hv1a/GNA或pro-Hv1a饲喂的幼虫未观察到死亡(结果未示)。

[0224] 表2:利用甘蓝夜蛾幼虫的口服喂食测定中重组毒素和融合蛋白的毒性

[0225]

	Pro-Hv1a	Hv1a/GNA	Pro-Hv1a/GNA	Hv1a (*大肠杆菌*)
存活百分数	85% (5d) 600 μg/g	70% (5d) 500 μg/g	10% (5d) 500 μg/g	100% (5d) 180 μg/g

[0226] 重组Hv1a、pro-Hv1a和pro-Hv1a/GNA融合蛋白对豌豆蚜(A.pisum)和麦蚜(Sitobion avenae)的摄取毒性

[0227] 通过以0.125mg-0.75mg/ml(125-750ppm)的浓度结合入人工饲料中来检测重组pro-Hv1a蛋白、pro-Hv1a/GNA和Hv1a/GNA针对豌豆蚜和麦蚜的口服活性。如对于鳞翅目幼虫所观察的，发现纯化的pro-Hv1a/GNA比Hv1a/GNA对两种蚜虫物种都显著更具毒性(图6A&B)。750ppm的Pro-Hv1a/GNA在3天后引起豌豆蚜虫100％死亡，而相同剂量的Hv1a/GNA在喂食8天后仅导致50％死亡。在500ppm的较低剂量，与Hv1a/GNA的20％的死亡率相比，以pro-Hv1a/GNA喂食的豌豆蚜虫在喂食8天后的死亡率是100％。

[0228] 还发现Pro-Hv1a/GNA比Hv1a/GNA对麦蚜显著的更具毒性。如图6B中所示，与对于Hv1a/GNA喂食的蚜虫60％的死亡率相比，用含有250ppm pro-Hv1a/GNA的饲料喂食7天的麦蚜记录到100％死亡率。麦蚜似乎比豌豆蚜虫对pro-Hv1a/GNA更易感，因为在低至125ppm pro-Hv1a/GNA的水平观察到显著的死亡率水平(2天喂食后80％死亡)，而对于以相同剂量

的融合蛋白喂食的豌豆蚜虫未记录到死亡。

[0229] 重组MODHv1a和Pro-Hv1a/GNA融合蛋白对麦蜈蚣(Deroceras reticulatum)的注射毒性

[0230] 通过注射入成体蜈蚣(～0.2g)检测MODHv1a/GNA和Pro-Hv1a/GNA对于蜈蚣(D.reticulatum)的活性。MODHv1a/GNA对应于修饰形式的Hv1a/GNA,其中Hv1a C-末端的单个氨基酸改变已经显示改善完整融合蛋白的表达,但具有与Hv1a/GNA相当的毒性。将蜈蚣在4℃冷却(～15分钟),之后注射重悬在20μl PBS中的25、50或100μg的纯化的Hv1a/GNA。每日评估死亡率达7天。图7显示对于两种处理观察到的剂量依赖性的死亡率。对于所有注射剂量,与MODHv1a/GNA相比,对于pro-Hv1a/GNA的死亡率显著更高($P<0.05$;Mantel Cox检验)。例如,与注射50μg MODHv1a/GNA后5天观察到的10％死亡率相比,注射50μg pro-Hv1a/GNA后3天记录到100％死亡率。

[0231] 研究PI1a的毒性的实验

[0232] 引文

[0233] 对于Hva1/GNA融合蛋白获得的结果通过选取其基因序列不包括预测的前区域的毒素蛋白并基于全球蛋白数据库中的相似序列将前区域结合入表达构建体中来延伸。使用来自蜘蛛阴暗拟隙蛛的毒素δ-amaurobitoxin-PI1a。

[0234] 重组PI1a和PI1a/GNA的表达和纯化

[0235] 用于在巴斯德毕赤酵母中生产重组蛋白的表达构建体基于载体pGAPZαB,其含有用于引导重组蛋白表达的强组成型启动子,并且其被设计为在GAPDH基因座整合入宿主基因组,得到稳定的转化子。用于生产重组Pl1a的表达构建体含有对应于被称为PI1a的毒素的公布的氨基酸序列的合成的编码序列,其排列在编码酵母α-因子前体前序列的序列的C-端框内。含有毒素前区域的构建体在酵母α-因子前体前序列和Pl1a毒素序列之间插入这些。使用的前区域取自来自蜘蛛Agelena orientalis的被称为Ao1bPro-PI1a的密切相关的毒素U3-agatoxin-Ao1b(Pl1a未获得包括前区域的cDNA序列),和取自被称为Hv1aPro-PI1a的如之前描述的Hv1a atracotoxin的前区域。表达构建体还含有编码myc表位$(His)_6$标签(由载体提供)的C-端序列。

[0236] 对于重组PI1a/GNA融合蛋白的生产,产生三种表达构建体,并且所有都含有经3氨基酸接头肽与对应于成熟雪花莲凝集素(GNA)的残基1-105的编码序列的N-末端融合的成熟PI1a编码序列;同样,融合蛋白框内排列在α-因子前体前序列的C-端和编码$(His)_6$标签的序列(由载体提供)的N-端。修饰的融合蛋白构建体还含有如上所述的Ao1b和Hv1a的前区域,其被插入在酵母α-因子前体前序列和Pl1a的成熟编码序列之间;它们称为Ao1bPro-Pl1a/GNA和Hv1aPro-Pl1a/GNA。通过限制性酶切-连接组装构建体并在克隆后通过DNA测序检查。

[0237] 将验证的表达构建体的克隆转化入蛋白酶-缺陷巴斯德毕赤酵母株SMD1168H,使用抗生素(博莱霉素)选择转化子。对于每种表达构建体获得大约50个抗性克隆。通过蛋白质印迹对摇瓶培养中生长的选择的克隆的培养物上清分析重组蛋白的生产,从而允许选择产生最高水平的PI1a和PI1a/GNA融合蛋白的克隆。不需要筛选大量转化的酵母克隆,因为大多数克隆表达重组蛋白,如通过培养物上清的蛋白质印迹上预期大小的免疫反应条带的存在判断的(结果未示)。

[0238] 对于各个构建体，选择小规模培养物中筛选的那些的表达最好的克隆用于通过平台发酵的大规模蛋白生产。通过镍亲和层析纯化培养物上清并且将洗脱峰通过透析脱盐并冻干。在优化前，重组蛋白的产率与其他融合蛋白相当；产生大约26mg/L Pl1a，大约32mg/L Ao1bPro-PI1a，大约21mg/L PI1a/GNA，大约32mg/L Ao1bPro-PI1a/GNA和大约13mg/L Hv1aPro-PI1a，如通过半定量分析的估计。

[0239] 通过SDS-PAGE和蛋白质印迹分析纯化的重组PI1a毒素(图8)。在SDS-PAGE凝胶上，由没有添加的前区域的构建体产生的重组毒素Pl1a跑胶为在大约18kDa的指定分子量处紧密靠近的两个条带(图8A)；两个条带都与抗-$(His)_6$抗体免疫反应。重组Pl1a(包括标签序列)的预测的分子量是6.87kDa。在凝胶、样品和电泳前使用的还原剂不同的情况下，毒素的双条带可重复，但被认为是凝胶系统的假象，可能是由于SDS对多肽的差的结合的结果。当相同样品用6M脲预处理时，Pl1a在14kDa的指定分子量处形成单一条带(图8B)；移动性的改变表明凝胶假象，并且单一条带表示产物的均匀性。含脲凝胶上的进一步分析得到Pl1a的单一条带，在不封闭半胱氨酸残基残基的情况下为～11kDa的指定分子量，并且在用碘乙酰胺处理以封闭半胱氨酸残基后为～9kDa；这些结果说明在"正常"条件下由于在电泳前或电泳期间残基二级结构和半胱氨酸残基之间的相互作用导致的不正确的分子量。N-末端测序验证了Kex2pGAPZαB切割位点的不完全加工，导致在N-末端的另外的谷氨酸和丙氨酸残基和7.07kDa的预测的产物重量。有趣的是，由结合前区域(Ao1bPro-PI1a)的修饰的构建体产生的Pl1a在"正常"SDS-PAGE条件下运行为在～9kDa处紧密靠近的双条带，有一些在较高分子量具有弥散条带的证据(图8C)。包括另外的前区域的肽的预测的分子量是8.6kDa。N-末端测序确认，前区域存在于蛋白产物中，并且在丙氨酸和Ao1b前区域异亮氨酸的第一残基之间发生切割，得到8.46kDa的预测分子量。

[0240] "正常"Pl1a/GNA融合蛋白(即源自不含有另外的前区域的构建体)在SDS-PAGE凝胶上分离为两个18和21kDa的指定大小的主蛋白蛋白(图9A)。18kDa蛋白，与抗-GNA抗体免疫反应(结果未示)在质量上对应于对于重组PI1a/GNA预测的蛋白(17.1kDa)。21kDa蛋白也与抗-GNA抗体免疫反应，并且与18kDa条带具有相同的N-端序列。用去糖基化酶PNGase F(其切割通过N-糖苷键连接于Asn残基的碳水化合物侧链)处理，去除该条带，而作为处理的结果，对于Pl1a/GNA融合蛋白"正确"条带的强度增加(图9B)。该结果提示，额外的条带是由于在合成和分泌期间巴斯德毕赤酵母"核心"糖基化融合蛋白。GNA不含有潜在的N-糖基化位点，但Pl1a毒素序列在Asn-35处含有潜在的N-糖基化位点(N-X-S/T)。单个条带的N-端序列确定为E-A-A-A-G-，如对于在翻译和从巴斯德毕赤酵母分泌期间去除酵母α-因子前体前区域后的融合蛋白所预期的。此外，与重组GNA(12.7kDa)类似的指定分子量处的小量条带(其与抗-GNA抗体免疫反应(结果未示))，提示在生产和纯化过程中发生融合蛋白向其成分的小量切割。完整PI1a/GNA融合蛋白与切割的GNA的比例估计为～30:1，如通过在SDS-PAGE凝胶上的考马斯蓝染色所判断的。

[0241] 源自含有另外的前区域序列的构建体的PI1a/GNA融合蛋白二者(即Ao1bPro-PI1a/GNA和Hv1aPro-PI1a/GNA)在SDS-PAGE凝胶上分离为两个大约17和21kDa的主要染色条带(图9C&D)。较小的17kDa蛋白在质量上对应于对于去除前区域后的Ao1bPro-PI1a/GNA和Hv1aPro-PI1a/GNA(16.94kDa)所预测的蛋白，提示在两种情况下，前区域在酵母细胞加工期间被去除。较大21kDa蛋白条带最可能表示糖基化的蛋白，如对于Pl1a/GNA所观察的。

Ao1bPro-PI1a/GNA和Hv1aPro-PI1a/GNA二者都表达为100％完整融合蛋白，没有SDS-PAGE和蛋白质印迹证据表明在毒素和GNA序列之间切割。

[0242] 重组PI1a和Ao1bPro-PI1a蛋白对菜蛾(甘蓝夜蛾)幼虫的注射毒性

[0243] 将甘蓝夜蛾的新羽化的第5龄幼虫(重～45-55mg)用纯化的重组蛋白注射以评价和比较毒素和融合蛋白的体内活性。图10A显示用PI1a注射后幼虫的存活，并且图10B显示注射相当剂量的PI1a和Ao1bPro-PI1a后的存活。用PI1a毒素注射的幼虫在1-2小时内全部显示无力的瘫痪(几乎不移动和几乎完全不进食)。在第一个24小时内观察到大多数死亡。瘫痪期后，一些昆虫显示逐步恢复并且能够从新开始进食。P11a的影响是剂量依赖性的，24小时后的死亡率范围从20μg毒素/昆虫的75％至1.25μg毒素/昆虫的20％。甚至以高剂量的毒素，在72小时后观察不到完全死亡。从这些测定，基于50mg的平均幼虫重量，对于重组P11a估计的LD_{50}(48小时)是4.1μg/昆虫或82μg/g昆虫(参见表3)。天然PI1a的文献LD_{50}值(相对于重组PI1a)是9.5μg/g。该文献值代表夜蛾(Spodoptera)(与甘蓝夜蛾相同昆虫目的物种)中的LD_{50}值。

[0244] 从修饰的表达构建体(包括来自U3-agatoxin-Ao1b的前区域)产生的重组P11a显示与P11a类似的毒性作用，但一致地在较低的剂量比由没有该额外序列的构建体产生的P11a更有效(图10B)。此外，在第一个24小时期间观察到Ao1bPro-PI1a对死亡率的主要影响，死亡率范围从10μg毒素/昆虫的80％至1.25μg毒素/昆虫的30％。在这些测定中，存在由修饰的构建体产生的毒素导致的死亡率继续增加至72小时的趋势并且最高的毒素剂量(10μg毒素/昆虫)在72小时导致100％死亡率。同时利用由未修饰的构建体产生的P11a进行的测定得到与之前的测定类似的结果，并且在相同测定中的两种样品之间的直接比较表明，当分析相同剂量存活曲线时，由未修饰的和修饰的构建体产生的P11a之间的差异统计学上显著(图10B)。对于从修饰的构建体(Ao1bPro-PI1a)产生的重组P11a估计的LD_{50}(48小时)是～1.0μg/昆虫，或基于50mg的平均幼虫重量为21μg/g昆虫；这相当于～4倍的毒性增加(参见表3)。

[0245] 表3：利用甘蓝夜蛾幼虫的注射生物测定中重组毒素和融合蛋白的毒性

[0246]

	PI1a (文献)	**PI1a**	**Ao1bPro-PI1a**	**PI1a/GNA**	**Ao1bPro-PI1a/GNA**	**Hv1aPro-PI1a/GNA**
LD_{50}	9.5 μg/g (夜蛾*属*)*	82 μg/g	21 μg/g	11 μg/g (28 μg/g 融合)	7.6 μg/g (19 μg/g 融合)	<5 μg/g (<12 μg/g 融合)

[0247] *未获得甘蓝夜蛾的数据

[0248] P11a/GNA融合蛋白在注射入甘蓝夜蛾幼虫时还引起瘫痪和死亡，并且比单独毒素显著更有作用(图11A)。当用1.25-10μg融合蛋白/昆虫(相当于0.50-4.0μg PI1a/昆虫，因为重组P11a的分子量是PI1a/GNA融合蛋白的分子量的～0.404)注射幼虫时，在所有剂量观察到显著的死亡，并且在最高剂量观察到在24小时后的完全死亡(图11A)。如利用P11a的，

在测定的第一个24小时内观察到大部分死亡，并且作用是剂量依赖性的，范围从10μg融合蛋白/昆虫的100％死亡率至1.25μg融合蛋白/昆虫的35％死亡率。融合蛋白在该最低剂量的死亡率在72h后增加至65％，而只注射1.25μg毒素的死亡率在24至72小时之间变化没有20％。从这些测定，对于重组Pl1a/GNA融合蛋白估计的LD_{50}(48小时)是1.4μg/昆虫，或28μg/g昆虫(基于50mg的平均幼虫重量)。LD_{50}相当于0.56μg Pl1a毒素/昆虫，使得Pl1a/GNA融合蛋白活性基于摩尔数是由未修饰的构建体产生的毒素的～7倍，并且活性是由修饰的Ao1b-Pro-PI1a构建体产生的毒素的～2倍。通过使用在72小时的死亡数值获得类似的比。相同剂量的毒素和融合蛋白产生的死亡率的直接比较表明，三种处理彼此不同，并且与对照不同，$p<0.0001$(ANOVA)。在所有这些测定中，经72小时未观察到对照注射的昆虫的死亡。

[0249] 如利用Pl1a毒素观察到的，将Ao1b前区域加入至Pl1a/GNA融合蛋白表达构建体导致具有增强的生物活性的蛋白产物(图11B)。源自该构建体的融合蛋白产物具有0.94μg/昆虫的估计的LD_{50}(48小时)，在所有剂量(除了最高的)具有增加死亡率。将来自pro-HV1a毒素的备选前区域加入至Pl1a/GNA表达构建体也增强了得到的融合蛋白的生物活性(图11C)；该蛋白具有<0.6μg/昆虫的估计的LD_{50}(48小时)，尽管总体死亡率值与pro-Ao1b-Pl1a/GNA融合蛋白类似。这些数据提示，通过在Pl1a/GNA的表达构建体中包含前区域可以获得两倍的毒性增加。

[0250] 重组PI1a/GNA、AoIbPro-PI1a/GNA和Pro-Hv1aPI1a/GNA蛋白对菜蛾(甘蓝夜蛾)幼虫的摄取毒性

[0251] 在利用第3期甘蓝夜蛾幼虫的小滴喂食测定中，也观察到与注射测定中观察到的相似的源自包含前区域的表达构建体的融合蛋白的毒性增加(图12)。摄取单个2μl含有20μg融合蛋白的小滴后，5天后的死亡率对于PI1a/GNA是40％；对于AoIbPro-PI1a/GNA是50％并且对于Hv1aPro-PI1a/GNA是70％(表4中总结的数据)。对于对照处理(其中幼虫用30μg毒素或GNA喂食)观察到最小的存活的减少(0-20％)，并且对照的存活曲线显著不同于融合蛋白处理。这提供进一步证据表明，将前区域加入至PI1a/GNA构建体导致增加的生物活性。对于注射研究，观察到使用Hv1a前区域导致相对于未修饰的PI1a/GNA融合蛋白的最大的毒性增强。

[0252] 表4:利用甘蓝夜蛾幼虫的口服喂食测定中的重组毒素和融合蛋白的毒性

[0253]

	PI1a (重组)	**Ao1bPro-Hv1a**	**PI1a/GNA**	**Ao1bPro-PI1a/GNA**	**Hv1aPro-PI1a/GNA**
存活百分数	90% (5d) 400 μg/g	80% (5d) 400 μg/g	60% (5d) 500 μg/g	50% (5d) 500 μg/g	30% (5d) 500 μg/g

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序列表

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食品及环境研究机构(FERA)

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<213> 蓝山漏斗网蜘蛛(Hadronyche versuta)

<400> 8

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1 5 10 15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
35 40 45

[0004]

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
50 55 60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65 70 75 80

Ser Leu Glu Lys Arg Glu Ala Glu Ala Ala Ala Glu Asp Thr Arg Ala
85 90 95

Asp Leu Gln Gly Gly Glu Ala Ala Glu Lys Val Phe Arg Arg Ser Pro
100 105 110

Thr Cys Ile Pro Ser Gly Gln Pro Cys Pro Tyr Asn Glu Asn Cys Cys
115 120 125

Ser Gln Ser Cys Thr Phe Lys Glu Asn Glu Asn Gly Asn Thr Val Lys

130 135 140

Arg Cys Asp Ala Leu Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn
145 150 155 160

Ser Ala Val Asp His His His His His His
165 170

<210> 9
<211> 238
<212> PRT
<213> 蓝山漏斗网蜘蛛(Hadronyche versuta)

<400> 9

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
1 5 10 15

[0005]

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
35 40 45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
50 55 60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65 70 75 80

Ser Leu Glu Lys Arg Glu Ala Glu Ala Ala Ala Ser Pro Thr Cys Ile
85 90 95

Pro Ser Gly Gln Pro Cys Pro Tyr Asn Glu Asn Cys Cys Ser Gln Ser
100 105 110

Cys Thr Phe Lys Glu Asn Glu Asn Gly Asn Thr Val Lys Arg Cys Asp
115 120 125

Ala Ala Ala Asp Asn Ile Leu Tyr Ser Gly Glu Thr Leu Ser Thr Gly
130 135 140

Glu Phe Leu Asn Tyr Gly Ser Phe Val Phe Ile Met Gln Glu Asp Cys
145 150 155 160

Asn Leu Val Leu Tyr Asp Val Asp Lys Pro Ile Trp Ala Thr Asn Thr
165 170 175

Gly Gly Leu Ser Arg Ser Cys Phe Leu Ser Met Gln Thr Asp Gly Asn
180 185 190

Leu Val Val Tyr Asn Pro Ser Asn Lys Pro Ile Trp Ala Ser Asn Thr
195 200 205

[0006]

Gly Gly Gln Asn Gly Asn Tyr Val Cys Ile Leu Gln Lys Asp Arg Asn
210 215 220

Val Val Ile Tyr Gly Thr Asp Arg Trp Ala Thr Gly Val Asp
225 230 235

<210> 10
<211> 263
<212> PRT
<213> 蓝山漏斗网蜘蛛(Hadronyche versuta)

<400> 10

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1 5 10 15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln

20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
35 40 45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
50 55 60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65 70 75 80

Ser Leu Glu Lys Arg Glu Ala Glu Ala Ala Ala Glu Asp Thr Arg Ala
85 90 95

Asp Leu Gln Gly Gly Glu Ala Ala Glu Lys Val Phe Arg Arg Ser Pro
100 105 110

[0007]

Thr Cys Ile Pro Ser Gly Gln Pro Cys Pro Tyr Asn Glu Asn Cys Cys
115 120 125

Ser Gln Ser Cys Thr Phe Lys Glu Asn Glu Asn Gly Asn Thr Val Lys
130 135 140

Arg Cys Asp Ala Ala Ala Asp Asn Ile Leu Tyr Ser Gly Glu Thr Leu
145 150 155 160

Ser Thr Gly Glu Phe Leu Asn Tyr Gly Ser Phe Val Phe Ile Met Gln
165 170 175

Glu Asp Cys Asn Leu Val Leu Tyr Asp Val Asp Lys Pro Ile Trp Ala
180 185 190

Thr Asn Thr Gly Gly Leu Ser Arg Ser Cys Phe Leu Ser Met Gln Thr
195 200 205

Asp Gly Asn Leu Val Val Tyr Asn Pro Ser Asn Lys Pro Ile Trp Ala
210 215 220

Ser Asn Thr Gly Gly Gln Asn Gly Asn Tyr Val Cys Ile Leu Gln Lys
225 230 235 240

Asp Arg Asn Val Val Ile Tyr Gly Thr Asp Arg Trp Ala Thr Gly Val
245 250 255

Asp His His His His His His
260

<210> 11
<211> 37
<212> PRT
<213> 阴暗拟隙蛛(Pireneitega luctuosa)

[0008] <400> 11

Gly Cys Leu Gly Glu Gly Glu Lys Cys Ala Asp Trp Ser Gly Pro Ser
1 5 10 15

Cys Cys Asp Gly Phe Tyr Cys Ser Cys Arg Ser Met Pro Tyr Cys Arg
20 25 30

Cys Arg Asn Asn Ser
35

<210> 12
<211> 151
<212> PRT
<213> 阴暗拟隙蛛(Pireneitega luctuosa)

<400> 12

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser

1 5 10 15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
35 40 45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
50 55 60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65 70 75 80

Ser Leu Glu Lys Arg Glu Ala Glu Ala Ala Ala Gly Cys Leu Gly Glu
85 90 95

[0009]

Gly Glu Lys Cys Ala Asp Trp Ser Gly Pro Ser Cys Cys Asp Gly Phe
100 105 110

Tyr Cys Ser Cys Arg Ser Met Pro Tyr Cys Arg Cys Arg Asn Asn Ser
115 120 125

Ala Leu Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala Val
130 135 140

Asp His His His His His His
145 150

<210> 13
<211> 164
<212> PRT
<213> 阴暗拟隙蛛(Pireneitega luctuosa)

<400> 13

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
1 5 10 15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
35 40 45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
50 55 60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65 70 75 80

[0010] Ser Leu Glu Lys Arg Glu Ala Glu Ala Ala Ala Ile Ser Tyr Glu Glu
85 90 95

Gly Lys Glu Leu Phe Gln Lys Glu Arg Gly Cys Leu Gly Glu Gly Glu
100 105 110

Lys Cys Ala Asp Trp Ser Gly Pro Ser Cys Cys Asp Gly Tyr Cys Ser
115 120 125

Cys Arg Ser Met Pro Tyr Cys Arg Cys Arg Asn Asn Ser Ala Leu Glu
130 135 140

Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His His
145 150 155 160

His His His His

<210> 14
<211> 244
<212> PRT
<213> 阴暗拟隙蛛(Pireneitega luctuosa)

<400> 14

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
1 5 10 15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
35 40 45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
50 55 60

[0011]

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65 70 75 80

Ser Leu Glu Lys Arg Glu Ala Glu Ala Ala Ala Gly Cys Leu Gly Glu
85 90 95

Gly Glu Lys Cys Ala Asp Trp Ser Gly Pro Ser Cys Cys Asp Gly Phe
100 105 110

Tyr Cys Ser Cys Arg Ser Met Pro Tyr Cys Arg Cys Arg Asn Asn Ser
115 120 125

Ala Ala Ala Asp Asn Ile Leu Tyr Ser Gly Glu Thr Leu Ser Thr Gly
130 135 140

Glu Phe Leu Asn Tyr Gly Ser Phe Val Phe Ile Met Gln Glu Asp Cys
145 150 155 160

Asn Leu Val Leu Tyr Asp Val Asp Lys Pro Ile Trp Ala Thr Asn Thr
165 170 175

Gly Gly Leu Ser Arg Ser Cys Phe Leu Ser Met Gln Thr Asp Gly Asn
180 185 190

Leu Val Val Tyr Asn Pro Ser Asn Lys Pro Ile Trp Ala Ser Asn Thr
195 200 205

Gly Gly Gln Asn Gly Asn Tyr Val Cys Ile Leu Gln Lys Asp Arg Asn
210 215 220

Val Val Ile Tyr Gly Thr Asp Arg Trp Ala Thr Gly Val Asp His His
225 230 235 240

[0012] His His His His

<210> 15
<211> 256
<212> PRT
<213> 阴暗拟隙蛛(Pireneitega luctuosa)

<400> 15

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
1 5 10 15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
20 25 30

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
35 40 45

```
Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
    50                  55                  60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65                  70                  75                  80

Ser Leu Glu Lys Arg Glu Ala Glu Ala Ala Ala Ile Ser Tyr Glu Glu
                85                  90                  95

Gly Lys Glu Leu Phe Gln Lys Glu Arg Gly Cys Leu Gly Glu Gly Glu
            100                 105                 110

Lys Cys Ala Asp Trp Ser Gly Pro Ser Cys Cys Asp Gly Cys Ser Cys
        115                 120                 125

Arg Ser Met Pro Tyr Cys Arg Cys Arg Asn Asn Ser Ala Ala Ala Asp
    130                 135                 140
```

[0013]

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Asn Ile Leu Tyr Ser Gly Glu Thr Leu Ser Thr Gly Glu Phe Leu Asn
145                 150                 155                 160

Tyr Gly Ser Phe Val Phe Ile Met Gln Glu Asp Cys Asn Leu Val Leu
                165                 170                 175

Tyr Asp Val Asp Lys Pro Ile Trp Ala Thr Asn Thr Gly Gly Leu Ser
            180                 185                 190

Arg Ser Cys Phe Leu Ser Met Gln Thr Asp Gly Asn Leu Val Val Tyr
        195                 200                 205

Asn Pro Ser Asn Lys Pro Ile Trp Ala Ser Asn Thr Gly Gly Gln Asn
    210                 215                 220

Gly Asn Tyr Val Cys Ile Leu Gln Lys Asp Arg Asn Val Val Ile Tyr
```

225 230 235 240

Gly Thr Asp Arg Trp Ala Thr Gly Val Asp His His His His His His
245 250 255

<210> 16
<211> 262
<212> PRT
<213> 阴暗拟隙蛛(Pireneitega luctuosa)

<400> 16

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
1 5 10 15

Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln
20 25 30

[0014]

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
35 40 45

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
50 55 60

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
65 70 75 80

Ser Leu Glu Lys Arg Glu Ala Glu Ala Ala Ala Glu Asp Thr Arg Ala
85 90 95

Asp Leu Gln Gly Gly Glu Ala Ala Glu Lys Val Phe Arg Arg Gly Cys
100 105 110

Leu Gly Glu Gly Glu Lys Cys Ala Asp Trp Ser Gly Pro Cys Cys Asp
115 120 125

Gly Phe Tyr Cys Ser Cys Arg Ser Met Pro Tyr Cys Arg Cys Arg Asn
130 135 140

Asn Ser Ala Ala Ala Asp Asn Ile Leu Tyr Ser Gly Glu Thr Leu Ser
145 150 155 160

Thr Gly Glu Phe Leu Asn Tyr Gly Ser Phe Val Phe Ile Met Gln Glu
165 170 175

Asp Cys Asn Leu Val Leu Tyr Asp Val Asp Lys Pro Ile Trp Ala Thr
180 185 190

Asn Thr Gly Gly Leu Ser Arg Ser Cys Phe Leu Ser Met Gln Thr Asp
195 200 205

Gly Asn Leu Val Val Tyr Asn Pro Ser Asn Lys Pro Ile Trp Ala Ser
[0015] 210 215 220

Asn Thr Gly Gly Gln Asn Gly Asn Tyr Val Cys Ile Leu Gln Lys Asp
225 230 235 240

Arg Asn Val Val Ile Tyr Gly Thr Asp Arg Trp Ala Thr Gly Val Asp
245 250 255

His His His His His His
260

<210> 17
<211> 8
<212> PRT
<213> 未知

<220>
<223> 人工的

〈400〉 17

Trp Ser His Pro Gln Phe Glu Lys
1 5

〈210〉 18
〈211〉 5
〈212〉 PRT
〈213〉 未知

〈220〉
〈223〉 单条带序列

〈400〉 18

Glu Ala Ala Ala Gly
1 5

[0016] 〈210〉 19
〈211〉 56
〈212〉 PRT
〈213〉 蓝山漏斗网蜘蛛(Hadronyche versuta)

〈400〉 19

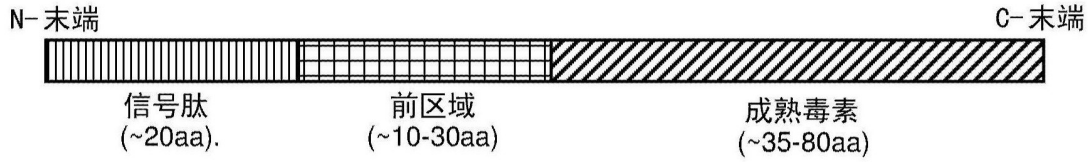
Glu Asp Thr Arg Ala Asp Leu Gln Gly Gly Glu Ala Ala Glu Lys Val
1 5 10 15

Phe Arg Arg Ser Pro Thr Cys Ile Pro Ser Gly Gln Pro Cys Pro Tyr
20 25 30

Asn Glu Asn Cys Cys Ser Gln Ser Cys Thr Phe Lys Glu Asn Glu Asn
35 40 45

Gly Asn Thr Val Lys Arg Cys Asp
50 55

A



B

EDTRADLQGGEAAEKVFRRSPTCIPSGQPCPYNENCCSQSCTFKENENGNTVKRCD

图 1：含有前区域的毒素的基因结构的示意图
(A) 含有前区域的毒素蛋白的基因结构的示意图。
(B) 蜘蛛毒素 Hv1a 的序列。加框的氨基酸序列对应于前区域。

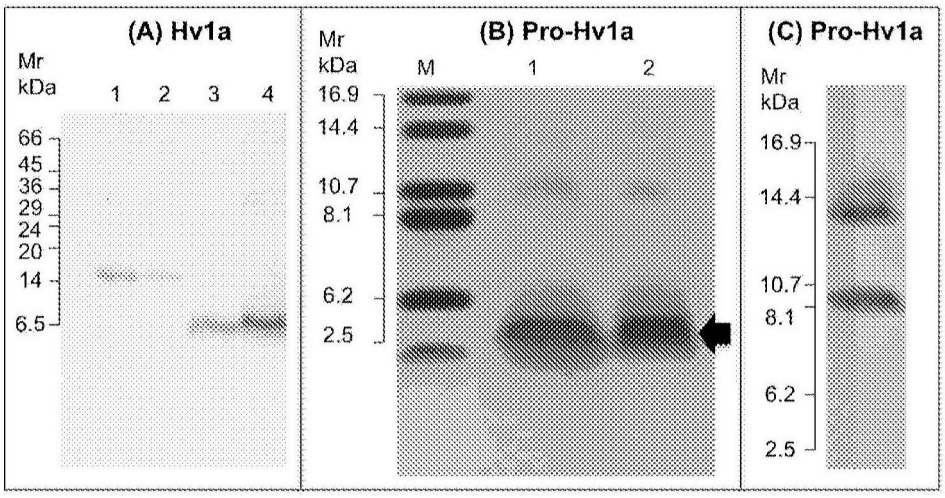


图 2: 重组 Hv1a 和 pro-Hv1a 毒素的表达和纯化 (A) SDS-PAGE 凝胶 (20% 丙烯酰胺) 分析, 其显示从培养物上清纯化重组的带 Strep 标签的 Hv1a 毒素。泳道 1 & 2 是 GNA 标准物 (分别为 0.5 和 0.25 μg) 并且泳道 3 & 4 是利用 2.5 mM 脱硫生物素从 Streptactin 柱洗脱后的峰级分 (10 μl)。(B) 重组 pro-Hv1a 的 Tris-Tricine 凝胶 (15% 丙烯酰胺) 分析, 泳道 1 & 2 是利用 0.2 M 咪唑从镍亲和柱洗脱后的峰级分 (10 μl)。箭头指示已经切除组氨酸标签的预测为 pro-Hv1a 的主要蛋白产物。(C) 使用抗 -His 抗体的 (B) 中样品的蛋白质印迹分析。

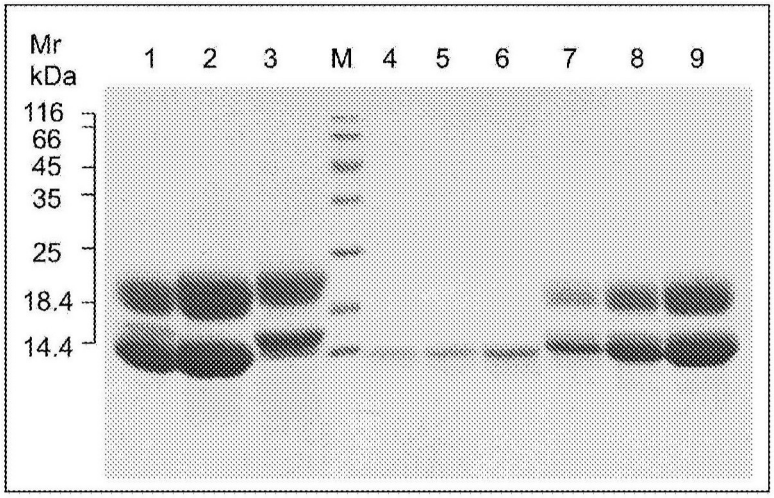
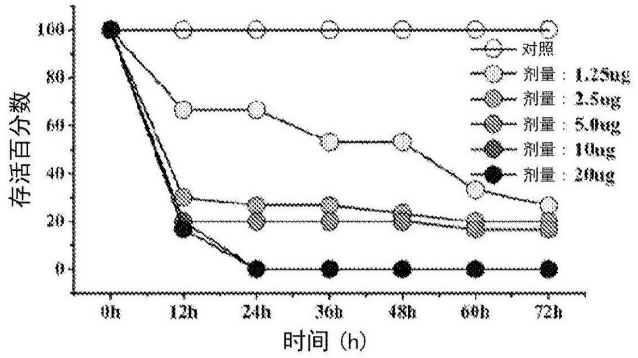
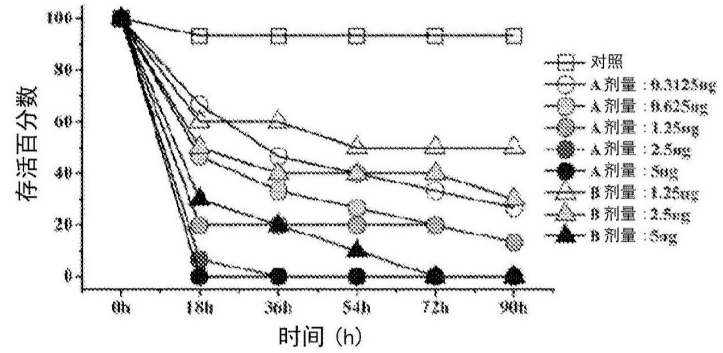


图 3:纯化的 pro-Hv1a/GNA、 Hv1a/GNA 和 MODHv1a/GNA 的冻干的样品的 SDS-PAGE 凝胶分析。 纯化的重组的含有 Hv1a 的融合蛋白的 SDS-PAGE 分析（17.5% 丙烯酰胺凝胶），用考马斯蓝进行针对总蛋白的凝胶染色。如下上样：泳道 1: pro-Hv1a/GNA;泳道 2:Hv1a/GNA; 泳道 3:MODHv1a/GNA;泳道 4-6:分别为 1、 2和 4 μg的 GNA 标准物； 泳道 7-9:12.5、 25 和 50 μg冻干的纯化的 pro-Hv1a/GNA （以使得能够定量融合蛋白含量）。

(A) Pro-Hv1a 第5龄幼虫



(B) Pro-Hv1a /GNA vs Hv1a/GNA 第3-4龄幼虫



(C) Pro-Hv1a /GNA vs Hv1a/GNA 第5龄幼虫

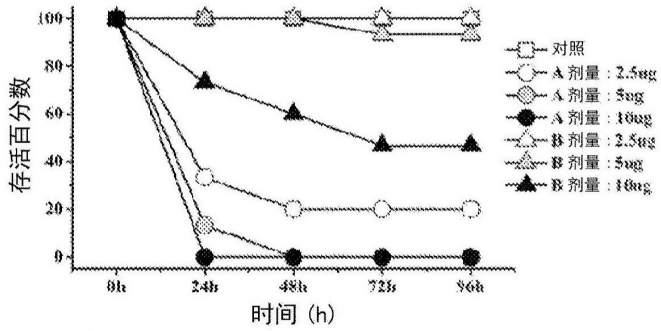


图4:重组pro-Hv1a、pro-Hv1a/GNA和Hv1a/GNA对甘蓝夜蛾(Mamestra brassicae)的注射毒性

注射不同剂量的重组pro-Hv1a、pro-Hv1a/GNA或Hv1a/GNA后第3-5龄甘蓝夜蛾幼虫的存活百分数。(A)注射各种剂量的pro-Hva1后第5龄幼虫的存活百分数。(B)注射各种剂量的pro-Hv1a/GNA(剂量A)或Hv1a/GNA(剂量B)后第3-4龄幼虫的存活百分数。(C)注射各种剂量的pro-Hv1a/GNA(剂量A)和Hv1a/GNA(剂量B)后第5龄幼虫的存活百分数。

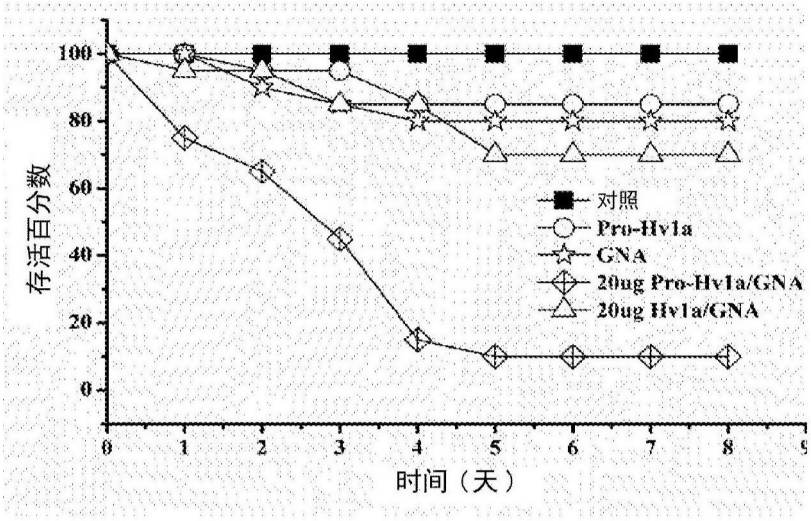


图5:重组pro-Hv1a、Hv1a/GNA、pro-Hv1a/GNA和GNA对甘蓝夜蛾的摄取毒性。摄取单次2 μl含有20 μg纯化的pro-Hv1a、Hv1a/GNA、pro-Hv1a/GNA或GNA的小滴后第3龄甘蓝夜蛾幼虫的存活百分数。对照幼虫以不含有添加的蛋白的小滴喂食（每次处理n=10）。

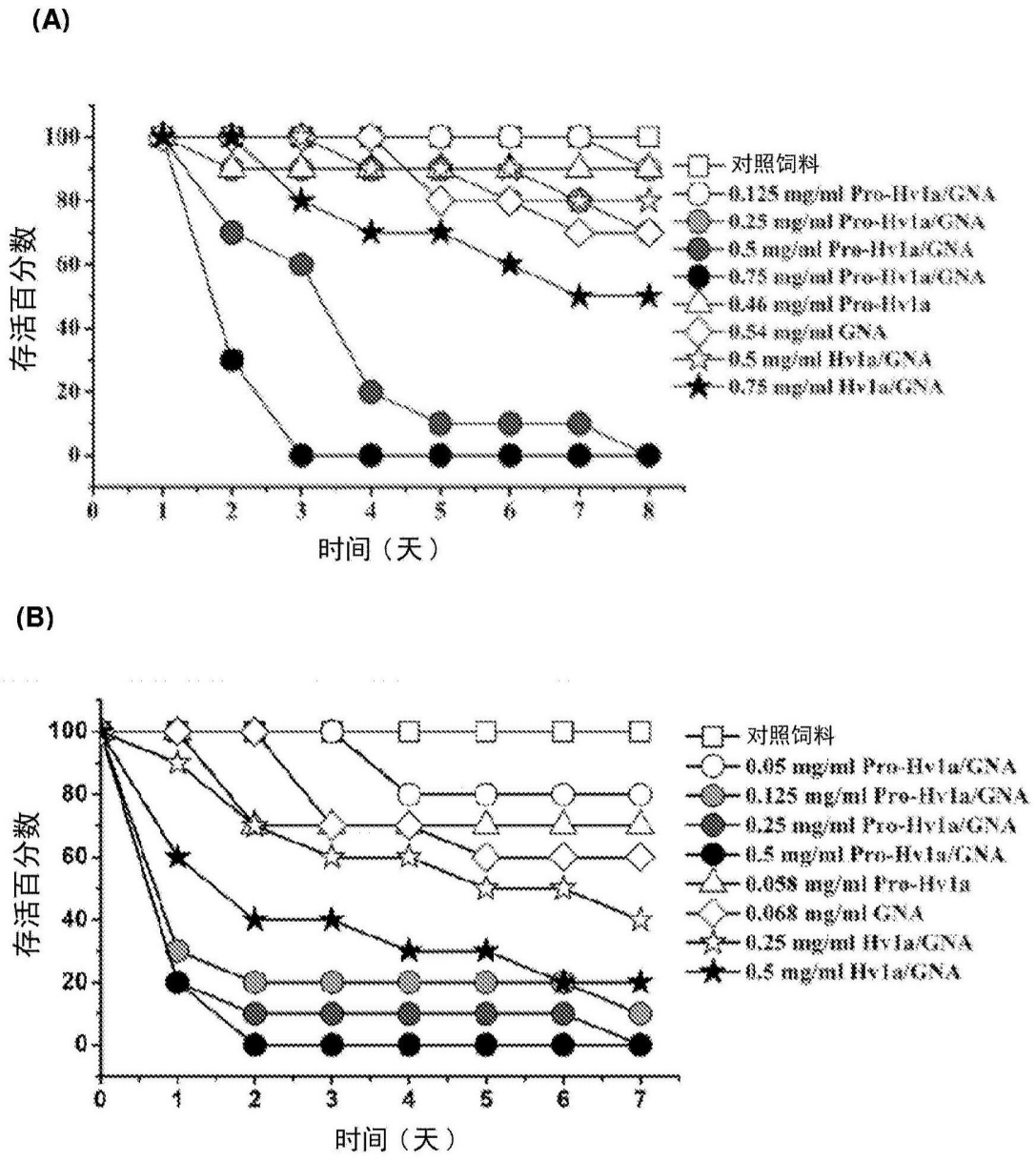


图 6: 重组 pro-Hv1a、 pro-Hv1a/GNA、 Hv1a/GNA 或 GNA 对豌豆蚜 (Acyrthosiphon pisum) 和麦长管蚜 (Sitobion avenae) 的摄取毒性 在含有 0.05 - 0.75 mg/ml 纯化的 重组 pro-Hv1a、 pro-Hv1a/GNA、 Hv1a/GNA 或 GNA 的人工饲料的情况下的 (A) 豌豆蚜（豌豆蚜虫）和 (B) 麦长管蚜（麦蚜）的存活百分数。

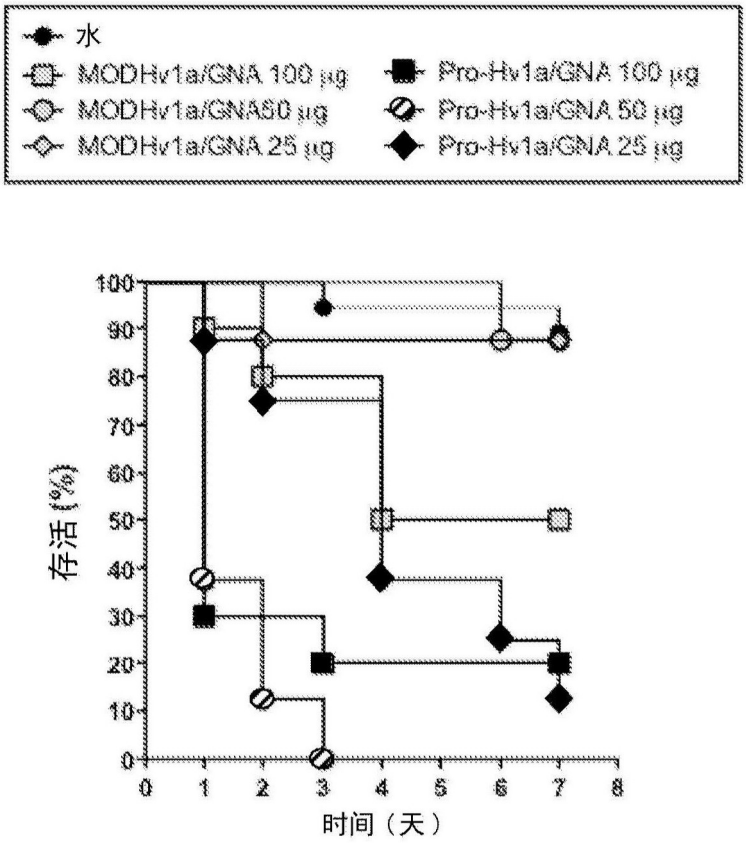


图 7: 重组 pro-Hv1a/GNA 和 Hv1a/GNA 对麦蛞蝓 (Deroceras reticulatum) 的注射毒性
被注射以 100、 50 或 25 μg 的 Hv1a/GNA 或 pro-Hv1a/GNA 的麦蛞蝓 (200 mg ± 40 mg) 的存活百分数。对于对照处理，n=18; 对于 100 μg 剂量，n=10; 并且对于 50 和 25 μg 剂量，n=8。

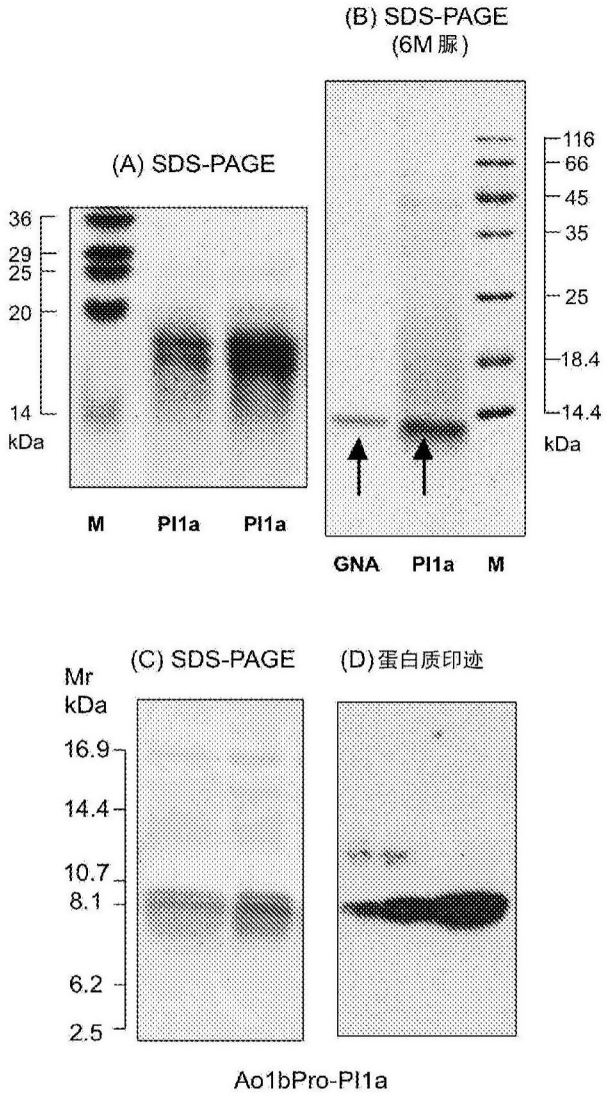


图 8: 重组 PI1a 和 Ao1bPro-PI1a 毒素的表达和纯化 (A) 在 " 普通 " SDS-PAGE 上分离的源自编码成熟毒素序列的构建体的 PI1a 毒素 ;M 表示标志物 , PI1a 的上样是 5 和 10 μg。(B) 用 6 M 脲变性后在 SDS-PAGE 上分离的 PI1a 毒素 (5 μg)。(C) 在 SDS-PAGE 上指定为 Ao1b 的源自含有前区域的构建体的重组 PI1a 毒素 , Ao1bPro-PI1a 的上样是 2.5 μg。(D) 使用抗 -His 抗体对纯化的 Ao1bPro-PI1a (25、 50 & 100 ng) 的蛋白质印迹。

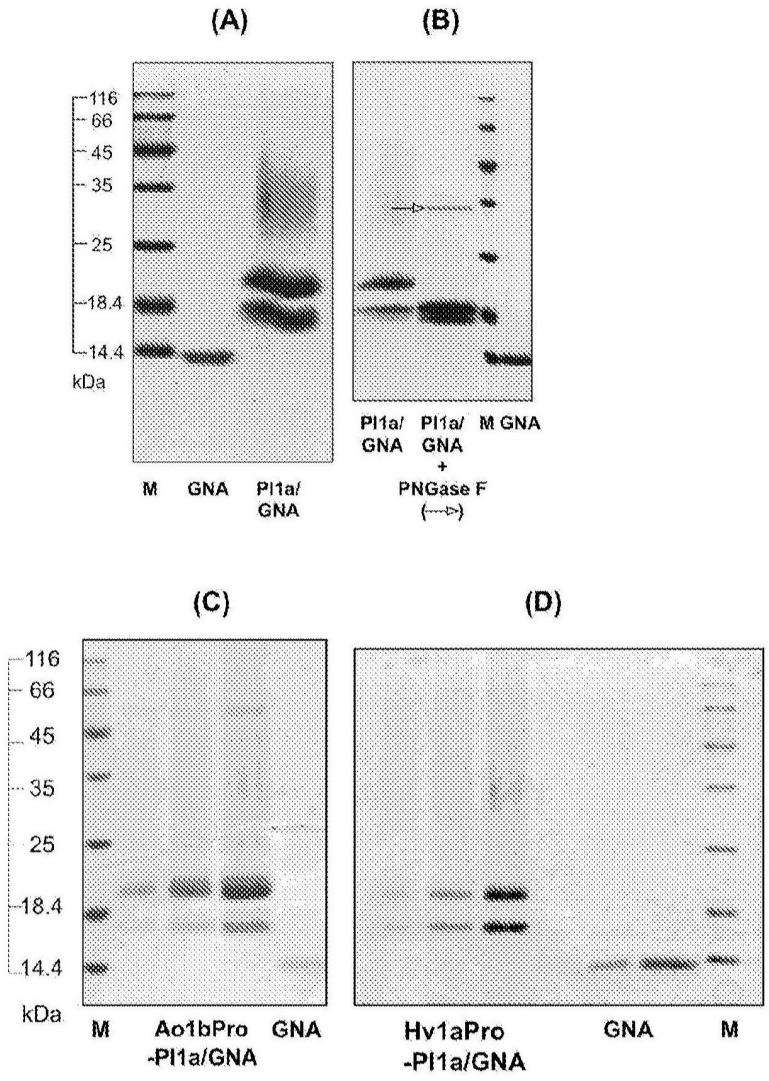
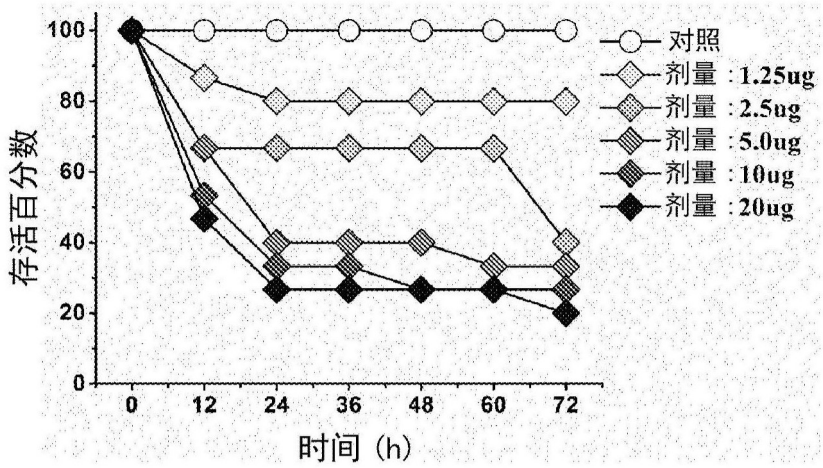


图 9:通过 SDS-PAGE 表征纯化的重组 PI1a/GNA 融合蛋白
(A) PI1a/GNA 融合蛋白 (10 μg) 和 GNA 标准物 (5 μg) 的 SDS-PAGE 分析。
(B) 使用 PNGase F 的 PI1a/GNA 融合蛋白的去糖基化 (空心箭头指示的条带), GNA 标准物 (5 μg)。(C) Ao1bPro-PI1a/GNA (1、2 和 4 μg) 的 SDS-PAGE 分析, GNA 标准物 (5 μg)。(D) Hv1aPro-PI1a/GNA (1、2 和 4 μg) 的 SDS-PAGE 分析, GNA 标准物 (1、2 和 4 μg)。

(A) PI1a 注射



(B) 注射**PI1a** 和 **Ao1bPro-PI1a** – 剂量 **A = PI1a;**剂量 **B = Ao1bPro-PI1a**

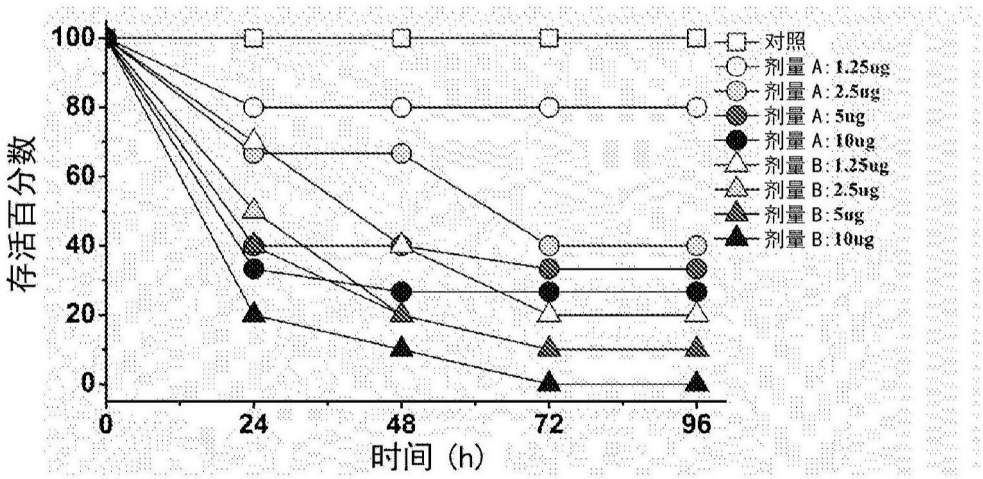


图 10: 重组 PI1a 和 Ao1bPro-PI1a 对甘蓝夜蛾的注射毒性
(A) 注射不同剂量的纯化的重组 PI1a 后第 5 龄甘蓝夜蛾幼虫的存活百分数。
(B) 注射不同剂量的纯化的重组 PI1a (剂量 A) 或 Ao1bPro-PI1a (剂量 B) 后第 5 龄甘蓝夜蛾幼虫的存活百分数。

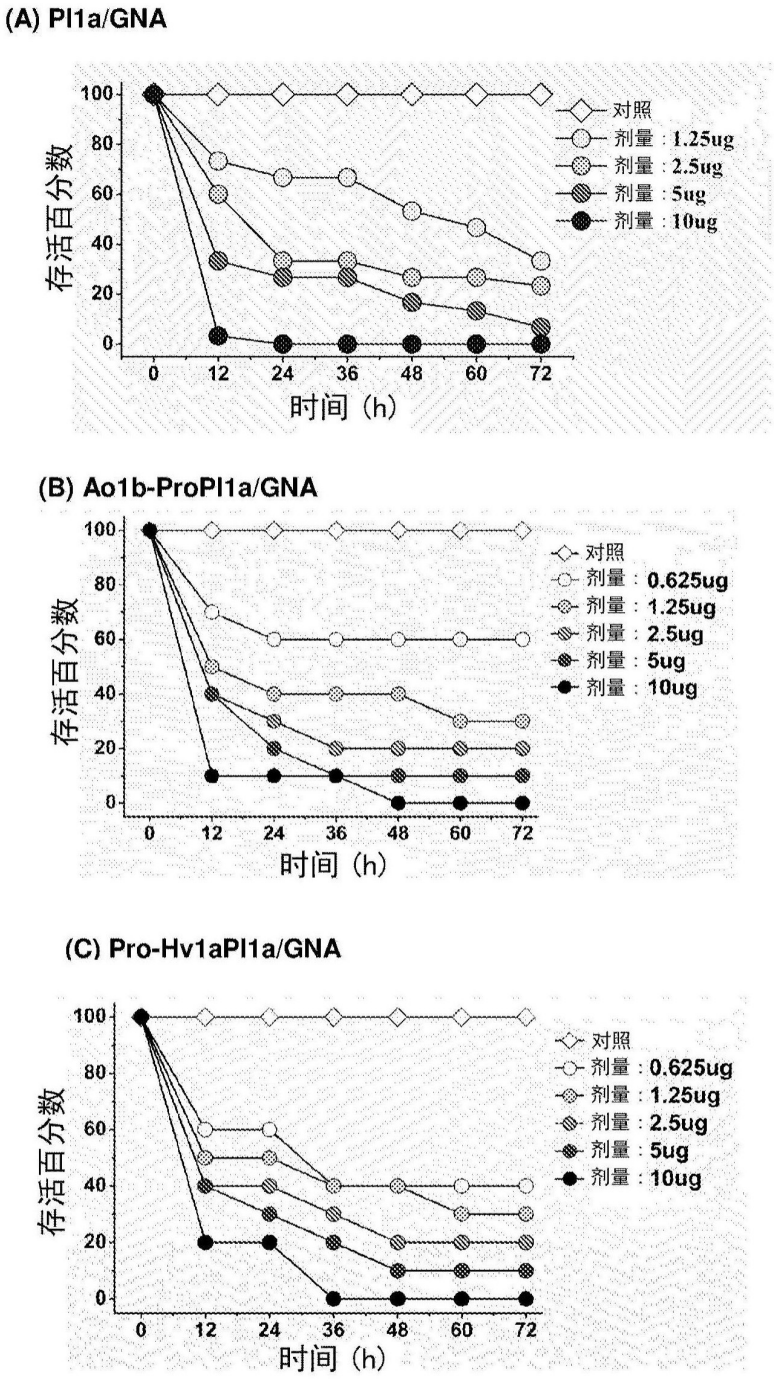
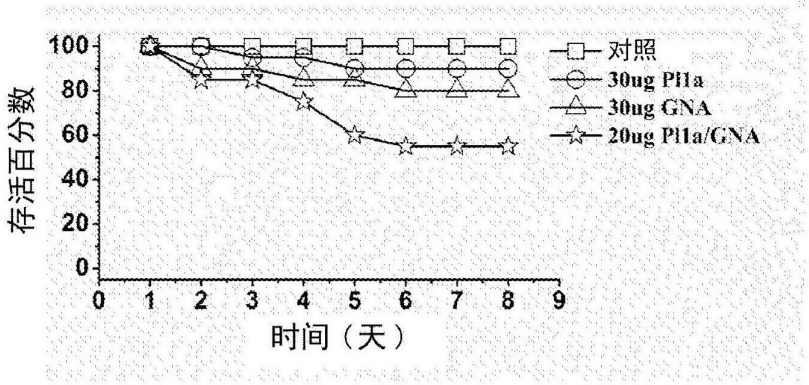
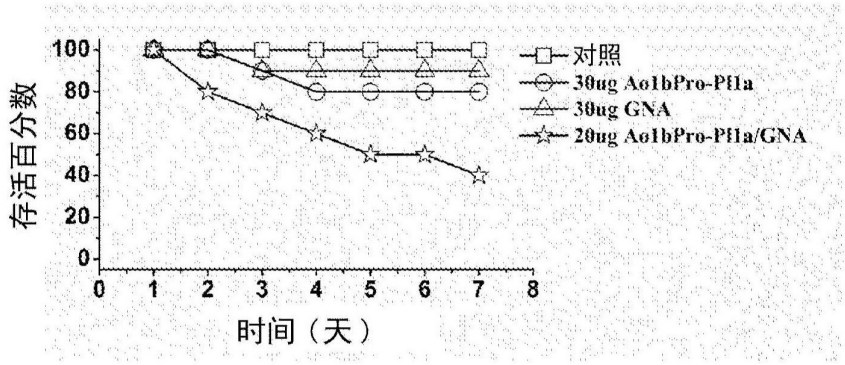


图 11：重组 PI1a/GNA、 Ao1bPro-PI1a/GNA 和 Pro-HV1a-PI1a/GNA 对甘蓝夜蛾的注射毒性 注射不同剂量的纯化的重组 PI1a/GNA （A）、 Ao1b-ProPI1a/GNA （B）或 Pro-Hv1a-PI1a/GNA （C）后第 5 龄甘蓝夜蛾幼虫的存活百分数。 每次处理 n=20 。

(A) 小滴饲喂：**PI1a/GNA**



(B) 小滴饲喂：**Ao1bPro-PI1a/GNA**



(C) 小滴饲喂：**Hv1aPro-PI1a/GNA**

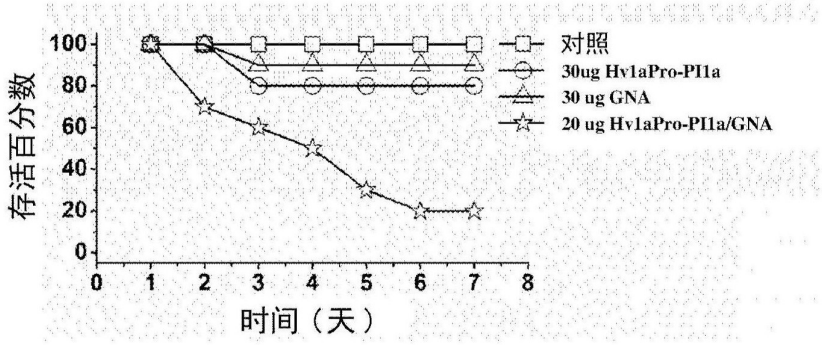


图 12: 重组 PI1a/GNA、 Ao1bPro-PI1a/GNA 和 Hv1aPro-PI1a/GNA 对甘蓝夜蛾的摄取毒性
摄取单次 2 μl 含有 20 μg 纯化的 PI1a/GNA (A)、Ao1bPro-PI1a/GNA (B) 或 Hv1aPro-PI1a/GNA (C) 融合蛋白的小滴后第 3 龄甘蓝夜蛾幼虫的存活百分数。在所有情况中，对照是单独的蔗糖（无添加的蛋白）；30 μg 的任一种 PI1a 毒素（成熟或修饰形式）或 GNA。