A *Bacillus thuringiensis* Cry protein controls soybean cyst nematode in transgenic soybean plants

Theodore W. Kahn1✉, Nicholas B. Duck1,2, Michael T. McCarville1, Laura Cooper Schouten1, Kathryn Schweri1, Jelena Zaitseva1 & Julia Daum1

Plant-parasitic nematodes (PPNs) are economically important pests of agricultural crops, and soybean cyst nematode (SCN) in particular is responsible for a large amount of damage to soybean. The need for new solutions for controlling SCN is becoming increasingly urgent, due to the slow decline in effectiveness of the widely used native soybean resistance derived from genetic line PI 88788. Thus, developing transgenic traits for controlling SCN is of great interest. Here, we report a *Bacillus thuringiensis* delta-endotoxin, Cry14Ab, that controls SCN in transgenic soybean. Experiments in *C. elegans* suggest the mechanism by which the protein controls nematodes involves damaging the intestine, similar to the mechanism of Cry proteins used to control insects. Plants expressing Cry14Ab show a significant reduction in cyst numbers compared to control plants 30 days after infestation. Field trials also show a reduction in SCN egg counts compared with control plants, demonstrating that this protein has excellent potential to control PPNs in soybean.
Plant-parasitic nematodes (PPNs) are major agricultural pests around the world, resulting in about $80 billion of damage per year. Losses in row crops are mostly due to cyst nematodes (Heterodera and Globodera spp.), root-knot nematodes (Meloidogyne spp.), and lesion nematodes (Pratylenchus spp.). Soybean cyst nematode (SCN), Heterodera glycines, is the most important pest of soybean in the United States, responsible for more than twice as much yield loss as the next greatest cause, and is one of the major pests of soybean in Brazil. H. glycines can reduce yield by 30–50%, even in fields with little or no visible above-ground symptoms. In the U.S., control of nematodes has relied on the use of varieties incorporating native resistance, crop rotation, and nematicidal seed treatments. One source of native resistance, derived from plant introduction (PI) accession 88788, results from a high copy number of the rhg1-b allele. PI 88788 is now used in 95–98% of H. glycines-resistant soybean varieties grown in the U.S., but its effectiveness against this pest has recently begun to decline.

Bacillus thuringiensis (Bt) is a Gram-positive sporulating bacterial species that produce a wide variety of insecticidal proteins, including the family of Cry (Crystal) proteins. Bt proteins are among the most broadly used natural insecticides in agriculture. Crops have been engineered to express Bt insecticidal proteins for controlling pests such as Lepidoptera and Coleoptera, generating commercial products that have delivered significant benefits to farmers over the past 25 years. Bt pesticides have a record of safety in agriculture stretching back more than half a century, with no significant risk to the environment or to human health. Some Bt proteins have been shown to be active against nematodes, but to date, there have been no transgenic crops on the market that control nematodes.

H. glycines is an obligate parasite that cannot easily be maintained or fed test substances in vitro. Caenorhabditis elegans has the advantage that it can easily be grown in vitro, and can be fed bacteria that express potential protein toxins, and can also be fed purified proteins. Genetically C. elegans has much in common with PPNs, although it lacks genes involved in parasitism. For these reasons, C. elegans is a useful surrogate for identifying proteins that will damage the nematode digestive system, and can potentially be expressed in transgenic crops to control nematodes in the same way that orally active insecticidal proteins have been used to control insect pests.

In this study, we describe a nematode-active Bt Cry protein, Cry14Ab, that is highly active against C. elegans. We show that Cry14Ab is somewhat unusual compared to many insecticidal Cry proteins in that the C-terminal half of the protein, commonly referred to as the crystallization region, is not easily removed in vitro by common proteases. However, in other ways, Cry14Ab is typical of Cry proteins in that it associates with the nematode intestine and causes damage to the intestine, consistent with the mechanism of action of insecticidal Cry proteins. Transgenic soybean events expressing Cry14Ab are protected from H. glycines in the greenhouse and field. The expression of the protein and the resistance to H. glycines is stable in F2 generations of the transgenic soybean plants, showing that Cry14Ab is a good candidate for commercial control of H. glycines in soybean.

**Results**

**Discovery of the strain.** Bacterial strains were isolated from soil and tested individually for activity against C. elegans in a liquid bioassay. C. elegans consumes whole bacteria, and will also consume soluble proteins. The bioassays included a laboratory E. coli strain (HB101) as worm food, as well as the bacteria or other material being tested for activity. A protein toxin need not be soluble in order to be active in this bioassay. Approximately 50 worms were placed in each well of a multi-well plate along with the material being tested for activity. Three to 5 days later, after the nematodes have had time to grow and reproduce, the wells were scored by eye under a dissecting microscope for the approximate number of nematodes present, the level of mobility of the nematodes, and the amount of clearing of worm food bacteria from the well. As shown in Fig. 1, a visual score from 0 to 5 was assigned to each well. A score of 0 was assigned to wells that resembled negative control wells, with hundreds of very active nematodes and almost complete clearing of worm food bacteria, indicating that the test material had no toxic effect on the nematodes. Higher score numbers were assigned when fewer and...
less active nematodes were observed, and when cloudiness from unconsumed worm food bacteria was present, indicating progressively stronger toxic effects of test substances. A score of 5 was assigned when only a few, sluggish worms were present, and the well was cloudy with unconsumed worm food bacteria, showing that the test material had inhibited the survival and reproduction of the nematodes.

Using this bioassay method, an active bacterial strain was identified. Bioassay scores of 3–5 were seen when this strain was grown for 1–5 days before being added to the bioassays. This bacterial strain was incapable of growing on nutrient agar containing a combination of tetracycline, chloramphenicol, and cefotaxime. When that combination of antibiotics was added to the C. elegans bioassays the results were the same as in the absence of the antibiotics, suggesting that activity against the nematodes may not have been dependent on active strain growth, and encouraging us to investigate the possibility that the strain may have released an active substance into the bacterial culture medium.

**Characterization of activity.** Cultures of the active bacterial strain were centrifuged to separate them into soluble and insoluble fractions. The soluble fractions were passed through 0.2 µm filters to remove any remaining cells, spores, crystals, or other insoluble material. The cell pellets were resuspended at the original concentration. The cell pellets showed activity (scores of 3–5) in bioassays. The sterile-filtered fractions were active when prepared from large cultures (30 mL) but not when prepared from small cultures (1 or 2 mL), showing that the cultures always contained insoluble active material, and sometimes contained soluble active material. When the insoluble pellet was extracted at pH 10 with dithiothreitol, the extract was active after being sterile filtered, showing that high pH could solubilize the insoluble active material. Spin filters with a 3 kDa molecular weight cutoff retained activity from sterile-filtered soluble samples, showing that the active moiety was larger than 3 kDa. No activity was seen (score of 0) when either the whole cultures, resuspended cultures, sterile-filtered culture supernatants, or sterile-filtered high pH extracts were heated to 95 °C for 10 min before being added to the bioassays, showing that the activity was heat-sensitive, and suggesting the possibility that a protein was responsible for the activity.

**Identification of strain.** The active strain was initially indicated to be Bacillus cereus by MIDI (The Sherlock™ Microbial Identification System) fatty acid analysis. DNA sequencing later showed the strain to be B. thuringiensis, with the taxonomic assignment being based on a whole-genome K-mer spectrum comparison to the NCBI RefSeq database, on 16S sequence, and on total genome average nucleotide identity compared to RefSeq. Microscopic observation revealed that the strain produces crystals (see Fig. 2a), which also indicated that the strain was Bt.

**Identification of active molecule.** To identify the molecule responsible for nematocidal activity in the Bt strain, a 100 mL culture was grown to sporulation and autolysis, the insoluble material was pelleted, and the supernatant was passed through a 0.2 µm filter to sterilize it. The sterile culture supernatant was spore-blotted to polyvinylidene fluoride (PVDF), and stained with Coomassie. Crystals are stained dark, while spores remain light. Photographs were taken once. Similar results were seen each time. Photographs were taken once. The active strain was initially indicated to be Bacillus thuringiensis (Bt) strain after autolysis, stained with Coomassie. Crystals are stained dark, while spores remain light.

Bacterial cultures were grown and observed microscopically for 5 days before being added to the bioassays. This bioassay method, an active bacterial strain was identified. Bioassay scores of 3–5 were seen when this strain was grown for 1–5 days before being added to the bioassays. This bacterial strain was incapable of growing on nutrient agar containing a combination of tetracycline, chloramphenicol, and cefotaxime. When that combination of antibiotics was added to the C. elegans bioassays the results were the same as in the absence of the antibiotics, suggesting that activity against the nematodes may not have been dependent on active strain growth, and encouraging us to investigate the possibility that the strain may have released an active substance into the bacterial culture medium.

**Characterization of activity.** Cultures of the active bacterial strain were centrifuged to separate them into soluble and insoluble fractions. The soluble fractions were passed through 0.2 µm filters to remove any remaining cells, spores, crystals, or other insoluble material. The cell pellets were resuspended at the original concentration. The cell pellets showed activity (scores of 3–5) in bioassays. The sterile-filtered fractions were active when prepared from large cultures (30 mL) but not when prepared from small cultures (1 or 2 mL), showing that the cultures always contained insoluble active material, and sometimes contained soluble active material. When the insoluble pellet was extracted at pH 10 with dithiothreitol, the extract was active after being sterile filtered, showing that high pH could solubilize the insoluble active material. Spin filters with a 3 kDa molecular weight cutoff retained activity from sterile-filtered soluble samples, showing that the active moiety was larger than 3 kDa. No activity was seen (score of 0) when either the whole cultures, resuspended cultures, sterile-filtered culture supernatants, or sterile-filtered high pH extracts were heated to 95 °C for 10 min before being added to the bioassays, showing that the activity was heat-sensitive, and suggesting the possibility that a protein was responsible for the activity.

**Identification of strain.** The active strain was initially indicated to be Bacillus cereus by MIDI (The Sherlock™ Microbial Identification System) fatty acid analysis. DNA sequencing later showed the strain to be B. thuringiensis, with the taxonomic assignment being based on a whole-genome K-mer spectrum comparison to the NCBI RefSeq database, on 16S sequence, and on total genome average nucleotide identity compared to RefSeq. Microscopic observation revealed that the strain produces crystals (see Fig. 2a), which also indicated that the strain was Bt.

**Identification of active molecule.** To identify the molecule responsible for nematocidal activity in the Bt strain, a 100 mL culture was grown to sporulation and autolysis, the insoluble material was pelleted, and the supernatant was passed through a 0.2 µm filter to sterilize it. The sterile culture supernatant was spore-blotted to polyvinylidene fluoride (PVDF), and stained with Coomassie. Crystals are stained dark, while spores remain light. Photographs were taken once. Similar results were seen each time. Photographs were taken once. The active strain was initially indicated to be Bacillus thuringiensis (Bt) strain after autolysis, stained with Coomassie. Crystals are stained dark, while spores remain light.

Bacterial cultures were grown and observed microscopically for 5 days before being added to the bioassays. This bioassay method, an active bacterial strain was identified. Bioassay scores of 3–5 were seen when this strain was grown for 1–5 days before being added to the bioassays. This bacterial strain was incapable of growing on nutrient agar containing a combination of tetracycline, chloramphenicol, and cefotaxime. When that combination of antibiotics was added to the C. elegans bioassays the results were the same as in the absence of the antibiotics, suggesting that activity against the nematodes may not have been dependent on active strain growth, and encouraging us to investigate the possibility that the strain may have released an active substance into the bacterial culture medium.

**Characterization of activity.** Cultures of the active bacterial strain were centrifuged to separate them into soluble and insoluble fractions. The soluble fractions were passed through 0.2 µm filters to remove any remaining cells, spores, crystals, or other insoluble material. The cell pellets were resuspended at the original concentration. The cell pellets showed activity (scores of 3–5) in bioassays. The sterile-filtered fractions were active when prepared from large cultures (30 mL) but not when prepared from small cultures (1 or 2 mL), showing that the cultures always contained insoluble active material, and sometimes contained soluble active material. When the insoluble pellet was extracted at pH 10 with dithiothreitol, the extract was active after being sterile filtered, showing that high pH could solubilize the insoluble active material. Spin filters with a 3 kDa molecular weight cutoff retained activity from sterile-filtered soluble samples, showing that the active moiety was larger than 3 kDa. No activity was seen (score of 0) when either the whole cultures, resuspended cultures, sterile-filtered culture supernatants, or sterile-filtered high pH extracts were heated to 95 °C for 10 min before being added to the bioassays, showing that the activity was heat-sensitive, and suggesting the possibility that a protein was responsible for the activity.

**Identification of strain.** The active strain was initially indicated to be Bacillus cereus by MIDI (The Sherlock™ Microbial Identification System) fatty acid analysis. DNA sequencing later showed the strain to be B. thuringiensis, with the taxonomic assignment being based on a whole-genome K-mer spectrum comparison to the NCBI RefSeq database, on 16S sequence, and on total genome average nucleotide identity compared to RefSeq. Microscopic observation revealed that the strain produces crystals (see Fig. 2a), which also indicated that the strain was Bt.

**Identification of active molecule.** To identify the molecule responsible for nematocidal activity in the Bt strain, a 100 mL culture was grown to sporulation and autolysis, the insoluble material was pelleted, and the supernatant was passed through a 0.2 µm filter to sterilize it. The sterile culture supernatant was spore-blotted to polyvinylidene fluoride (PVDF), and stained with Coomassie. Crystals are stained dark, while spores remain light. Photographs were taken once. Similar results were seen each time. Photographs were taken once. The active strain was initially indicated to be Bacillus thuringiensis (Bt) strain after autolysis, stained with Coomassie. Crystals are stained dark, while spores remain light.
cally, it is 87% identical to Cry14Aa at the amino acid level. It was against *C. elegans* falls into the phylogenetic family of Cry toxins known to be active on sequence alignment it is a 3-domain delta-endotoxin, and it a molecular weight of 132 kDa (GenBank: AGU13817.1). Based

Description of Cry14Ab

The protein has 1185 amino acids, with a molecular weight of 132 kDa (GenBank: AGU13817.1). Based on sequence alignment it is a 3-domain delta-endotoxin, and it falls into the phylogenetic family of Cry toxins known to be active against *C. elegans*, including Cry5, Cry14, and Cry21. Specifically, it is 87% identical to Cry14Aa at the amino acid level. It was assigned the name Cry14Ab by the Bacterial Pesticidal Protein Resource Center (https://www.bpprc.org/).

Heterologous expression in bacteria. The full-length PCR product of the Cry14Ab gene was inserted into a vector for expression in *E. coli* with an N-terminal 6his-maltose-binding protein tag, and into a vector for expression in a plasmid-cured strain of *Bt* with no tag. In *E. coli* the fusion protein was soluble at pH 8 and pH 10.5. In *Bt* the protein formed crystals with a similar appearance to the crystals in the native strain (Fig. 2b, above), but some soluble protein was also present in the culture supernatant, as was the case with the native strain. This similarity leads us to suspect that the crystals produced by the native strain also contain Cry14Ab, although we did not analyze those crystals.

**EC$_{50}$ of Cry14Ab.** The activity level of the native strain carrying the Cry14Ab gene, and of the purified protein itself, were measured using the liquid *C. elegans* bioassay described above. A dilution series of the native *Bt* strain carrying the Cry14Ab gene was fed to *C. elegans* as both the whole bacterial culture and the sterile-filtered culture supernatant (Fig. 3a). Both showed activity, indicating that the active species is partially soluble under the bacterial culturing conditions (~pH 8). Dilution caused the activity of the sterile supernatant to drop faster than the activity of the whole culture, showing that a significant amount of the active moiety is present in an insoluble form, perhaps as crystals still contained within intact bacterial cells or released by autolysed cells.

A dilution series of purified Cry14Ab expressed heterologously in *E. coli* as a fusion with maltose-binding protein (Fig. 3b), or untagged in an acryliforiferous (plasmid-cured) *Bt* strain (Fig. 3c), was fed to *C. elegans*, and the approximate effective concentration giving a response halfway between a score of 0 and 5 (EC$_{50}$) was calculated. Protein from the two sources gave approximately the same EC$_{50}$, with a value of about 7 µg/mL for the protein expressed in *E. coli* (taking into account only the mass of the Cry14Ab portion of the fusion protein) and about 11 µg/mL for the protein expressed heterologously in *Bt*. Other nematicidal toxins have roughly similar levels of activity against *C. elegans*, including Cry14Aa (16 µg/mL), Cry21Aa (47 µg/mL), and Cry5Ba (66 µg/mL).

**Cry14Ab is not easily processed to a protease-resistant core.** Cry proteins that are toxic to insects are typically solubilized in the insect gut and then cleaved by gut proteases to a protease-resistant core. In lepidopteran insects, trypsin-like or
This shows that Cry14Ab is not easily cleaved by chymotrypsin-like proteases, while the other proteases had no effect under the conditions used. Tilisin and proteinase K largely destroyed the protein at pH 10.4, while thermolysin, bromelain, and actinase E, at pH 5.0, pH 7.5, and pH 10.4, were less effective. Note that although pepsin was tested in these experiments, it is not active at the pH values used here. As seen in Fig. 4, subtilisin and proteinase K largely destroyed the protein at pH 10.4, while the other proteases had no effect under the conditions used. This shows that Cry14Ab is not easily cleaved by a protease-resistant 55–65 kDa core, which is not required for the damage to occur. This type of damage is similar to that seen when Cry5Ba, Cry14Aa, or Cry21Aa is fed to C. elegans, and it suggests that Cry14Ab kills C. elegans by damaging the intestine, which is also the mechanism by which other Cry toxins kill insects.

Effect of Cry14Ab on the intestine of C. elegans. To observe the effect of Cry14Ab on the intestine of C. elegans, nematodes were fed fluorescently labeled full-length Cry14Ab (132 kDa), fluorescently labeled truncated Cry14Ab (77 kDa), or fluorescently labeled bovine serum albumin (BSA; 66 kDa) for 24 h. The fluorescent label allowed us to ensure that the nematodes had actually ingested the protein. As a first step, we determined that the labeling had not destroyed the activity of Cry14Ab by performing a single C. elegans bioassay on a dilution series of labeled full-length and truncated Cry14Ab (the experiment was not repeated, and precise EC50 values were not determined). The results showed that labeled Cry14Ab had about the same level of toxicity as unlabeled protein (see Fig. 5, full-length unlabeled and full-length labeled), while BSA was not toxic. We then fed the labeled proteins to nematodes and observed the worms by microscopy (Fig. 6a through c). Under white light, the BSA-fed worms appeared normal, similar to worms that were incubated with excess labeled protein, and imaged by fluorescence microscopy. In the case of both labeled Cry14Ab and labeled BSA, the lumen of the intestine was seen to be filled with labeled protein (Fig. 6d).
To determine if Cry14Ab was being retained in the intestine, ~50 worms were fed labeled protein for 24 h, rinsed to remove excess labeled protein, and placed in a medium without labeled protein for another 24 h, after which about 75% of the worms were still alive. Worms that had been fed labeled BSA showed no fluorescence in the intestine, but all surviving worms that had been fed labeled Cry14Ab retained some fluorescence, showing that the protein remained associated with the intestine (Fig. 6e).

Similar results were seen when the worms were fed the fluorescently labeled purified N-terminal region of trypsin-truncated G708R protein, showing that the C-terminal portion of the protein is not needed for association with intestinal cells in C. elegans. The retention of Cry14Ab is consistent with the fact that 3-domain delta-endotoxins bind to receptors on the surface of intestinal cells.29

Induced ingestion of Cry14Ab by H. glycines. At this point, we switched to working with H. glycines, the pest of interest. H. glycines is an obligate parasite that does not normally feed until it has established specialized feeding sites within roots,16 but newly hatched second-stage juveniles (J2) that have not yet entered roots can be induced to ingest substances dissolved in a liquid by adding the neurotransmitter octopamine to the liquid.30 To determine if J2 H. glycines nematodes could ingest a 132 kDa protein, fluorescently labeled full-length Cry14Ab at a concentration of 1 mg/mL was fed to the nematodes in liquid, using octopamine to stimulate ingestion. The fluorescently labeled protein was seen in the esophagus and intestine, showing that a protein of this size can pass through the nematode’s stylet (Fig. 7a). No fluorescence was observed in nematodes that had not been fed fluorescently labeled protein (Fig. 7b).

Greenhouse evaluation of efficacy of Cry14Ab against H. glycines in soybean. Soybean cv. Jack was transformed with Cry14Ab. Jack, which was released in 1989 as an H. glycines-resistant variety,31 carries a high copy number of the rgh1-b allele from PI 88788. Jack is not currently used commercially because of its relatively low yield, but it was chosen for this study because plants can easily be regenerated after being transformed with foreign genes using the aerosol beam transformation method.32
Cry14Ab soybean events described in this study were generated by co-transforming plants with two separate vectors that contained the cry14Ab1 gene on one vector, and the selectable marker herbicide resistance gene grg23ace5 on the other (Supplementary Fig. 2). The presence of the cry14Ab gene in regenerated transformed plants was confirmed by PCR using primers Cry14Ab_p2f and Cry14Ab_p3r (see Supplementary Table 1), which amplify a fragment of the gene, and its expression was confirmed by western blot. Plants that contained the gene and had a detectable expression of the protein were allowed to self, and the seed was used in greenhouse and field H. glycines efficacy testing.

T2 homozygous plants showed consistent levels of accumulation of the Cry14Ab protein (Fig. 8a). A second blot of another 9 plants showed similar results. H. glycines-infested roots of soybean negative control event SB172, stained pink with acid fuchsins 10 days post infestation. Four plants were imaged, of which one is shown. H. glycines-infested roots of soybean Cry14Ab event SB166, stained pink with acid fuchsins 10 days post infestation. Four plants were imaged, of which one is shown. Source data are provided as a Source Data file.

Fig. 7 Microscopic observation of ingestion of fluorescently labeled Cry14Ab by Heterodera glycines. a Two second-stage juvenile H. glycines nematodes were fed IR680-labeled Cry14Ab at 1 mg/mL in the presence of the feeding stimulant octopamine for 24 h. Each nematode is shown under white light on the left, and with a Cy5 filter set on the right. The experiment was performed twice with 200 nematodes under each condition. About half ingested labeled protein, and three representative nematodes were photographed each time, of which two are shown here. b A nematode that was not fed labeled Cry14Ab, under white light on the left, and with a Cy5 filter set on the right. Source data are provided as a Source Data file.

Fig. 8 The ability of Cry14Ab to reduce Heterodera glycines infection in roots of plants grown in the greenhouse was assessed. a Western blot of 10 individual T2 homozygous plants from event SB166, showing consistent accumulation of Cry14Ab protein. A second blot of another 9 plants showed similar results. H. glycines-infested roots of soybean negative control event SB172, stained pink with acid fuchsin 10 days post infestation. Four plants were imaged, of which one is shown. c H. glycines-infested roots of soybean Cry14Ab event SB166, stained pink with acid fuchsin 10 days post infestation. Four plants were imaged, of which one is shown. Source data are provided as a Source Data file.
Cry14Ab: n OP50, and after 60 days adult females and cysts were counted, giving FI the presence of native resistance. Using the standard methods34, populations, both of which are considered virulent to PI 88788-we assessed the ef degree of expressed as FI with FI < 10 were categorized as highly resistant to selection vector (pAX5219). The results are shown in Fig. 9. The segregants, or (3) negative control events transformed only with a glycines expressing events (Cry14Ab). Soybean plants were grown in sand in 4-inch clay pots in a greenhouse and infested at 2 weeks with H. glycines. Untransformed plants and negative control events transformed only with a check cultivar in our greenhouse assays. Jack contains native resistance contributed by Cry14Ab was resistant to H. glycines derived from PI 88788, and using it as the field population of H. glycines-recessive cultivars, which call for a minimum of three replications and two years of data37. The site was chosen based on the presence of a H. glycines population virulent to PI 88788-derived resistance in the Jack soybean variety. Five Cry14Ab events were planted along with two control treatments.

Standard greenhouse methods have been developed to evaluate new soybean varieties for H. glycines resistance. In a standard assay, the degree of reproduction of H. glycines, known as the female index (FI), is measured by the ability of female nematodes to grow and reproduce on a standard H. glycines-susceptible soybean check cultivar (typically Lee 74, Essex, Hutcheson, or Williams 82) and on the test cultivar, grown and infested in parallel. The FI value is calculated from at least three replicated results using the formula: (mean number of females on test cultivar)/(mean number of females on check cultivar)×100. By convention cultivars with a FI < 10 are considered resistant, and those with a FI between 10 and 30 are considered moderately resistant. We utilized the transformation background Jack as the check cultivar in our greenhouse assays. Jack contains native resistance to H. glycines derived from PI 88788, and using it as the check cultivar allowed us to measure the efficacy of Cry14Ab in the presence of native resistance. Using the standard methods34, we assessed the efficacy of Cry14Ab against two H. glycines populations, both of which are considered virulent to PI 88788-derived resistance. The first population was an inbred line OP5035 (HG type36 1.2.3.5.6), and the second was a field population of HG type 2.5.7 H. glycines. while events with FI of 10–30 were categorized as moderately resistant to H. glycines34. Note that many commercially available soybean varieties labeled as H. glycines resistant do not meet the FI < 10 standard in greenhouse testing, so while this value can indicate H. glycines resistance, results in the field appear to depend on many additional factors.

In this study, seven independent Jack events that had a consistent expression of Cry14Ab were produced and were tested as described above to determine if Cry14Ab was efficacious against H. glycines. Events that accumulated Cry14Ab protein based on western blot analysis had significantly fewer H. glycines females on their roots than plants that did not contain the Cry14Ab gene (either Cry14Ab-negative segregants, untransformed Jack, or negative control events SB171 or SB172 that had been transformed with pAX5219 only). Cry14Ab reduced infection by both the OP50 and HG type 2.5.7 H. glycines populations. To test whether the Cry14Ab trait was stable in the T2 soybean generation, the H. glycines greenhouse assays were repeated using plants grown from segregating T2 hemizygous seed from some events. The results revealed that the gene was transferred to the next generation, and continued to provide resistance to H. glycines.

Field evaluation of efficacy of Cry14Ab against H. glycines in soybean. Field trials were conducted in Webster County, Iowa in 2010 and 2011. We used standard practices for field evaluations of H. glycines-resistant cultivars, which call for a minimum of three replications and two years of data37. The site was chosen based on the presence of a H. glycines population virulent to PI 88788-derived resistance in the Jack soybean variety. Five Cry14Ab events were planted along with two control treatments.
The control treatments consisted of untransformed Jack, and Jack transformed with the herbicide tolerance gene (gyp23ace5) alone. The experimental treatments were planted in three-row plots arranged in a randomized complete block with four replications. Soil samples were collected from each experimental plot to determine the initial, mid-season, and end-of-season *H. glycines* egg population densities in all plots. A reproductive factor was calculated to evaluate nematode reproduction mid-season and end-of-season. Reproduction factors were calculated by dividing the population density at the assessment time by the population density at planting. The natural log of the reproductive factor plus one is displayed. Data were natural log (ln) transformed and analyzed in an ANOVA. Contrast statements were used to compare control and Cry14Ab treatments with an F-test. Cry14Ab-expressing soybean reduced *H. glycines* reproduction at both the mid-season (*P* = 0.00179) and end-of-season (*P* = 0.00156) assessment times. In examining non-transformed data, mid-season reproduction was reduced by 43%, from a reproduction factor of 1.25 ± 0.8–0.71 ± 1.06 and end-of-season reproduction was reduced by 60%, from a reproduction factor of 1.67 ± 2.52–0.67 ± 1.0. Box plots depict the median (middle line), average (x), 25th and 75th percentile (box), and 5th and 95th percentile (whiskers) as well as all data points. Source data are provided as a Source Data file.

The mechanism of action of Cry14Ab is likely to be fundamentally different from the putative mechanism of the native trait found in PI 88788, which is believed to work by interfering with vesicular trafficking in feeding sites, preventing the nematodes from continuing to feed15. By contrast Cry14Ab most likely directly damages the intestines of the nematodes. This can explain why Cry14Ab controls *H. glycines* HG types that are virulent to PI 88788. Combining Cry14Ab with PI 88788 in soybean plants should delay the development of virulence in *H. glycines* to both of these control mechanisms.

Soybean farmers are in need of new methods to control *H. glycines*, one of the principal causes of damage in the crop. Expression of Cry14Ab in soybean plants is a potential approach to controlling this pest, and as such may help farmers deal with the growing virulence of *H. glycines* to existing control methods.
Methods

Bacterial strain isolation from environmental samples. Environmental samples of soil were collected from many locations. Soil samples were plated onto the surface of Luria Bertani (LB) agar and individual colonies were selected and transferred onto fresh LB agar. Additional re-streaks were performed until visually uniform, purified strains were obtained. Liquid cultures of purified strains were stored at −80 °C, and these frozen stocks were used for strains that showed activity in C. elegans bioassays.

C. elegans bioassays. Standard methods were used to maintain N2 C. elegans cultures as described in WormBook.22 Nematodes were maintained on MYOB C. elegans containing protein were pooled and dialyzed against 20 mM Tris pH 8.0 overnight. The dialysis was eluted with 5 column volumes of Buffer A with 10 mM maltose. Fractions was loaded onto the column through a sample loop at a flow rate of 0.5 mL/min. Lysates were then purified using cobalt resin. The mutant protein was expressed in Bt as for wild-type protein and was cleaved with trypsin. The N-terminal fragment of the cleaved protein was purified by anion exchange chromatography and was of the expected size based on SDS–PAGE, and an aliquot was fluorescently labeled as for wild-type protein.

Proteolysis of Cry14Ab. Cry14Ab was expressed in a plasmid-cured strain of Bt grown in liquid medium to sporulation and then pelleted. The spore/crystal pellets were reconstituted with 20 mM CAPS pH 10.5, 10 mM beta-mercaptoethanol, 10% glycerol, and the supernatant was run on a Superdex 200 gel filtration column in the same buffer. Fractions containing Cry14Ab were pooled and concentrated using Centricon centrifugal filter units with a molecular weight cutoff of 10 kDa. Purified protein at 0.4 mg/mL was incubated with 0.004 mg/mL of each protease (1:100 w/w ratio of protease to Cry14Ab) using Hampton Research Proti-Ace and Proti-Ace 2 kits, in 20 mM sodium acetate pH 5.1, 0.15 M NaCl, or 5 mM HEPES pH 7.5, 0.25 M NaCl, or 20 mM CAPS pH 10.4, 0.15 NaCl in 2 H2O at 37 °C. The reactions were halted by heating to 95 °C for 10 min in SDS–PAGE sample buffer with β-mercaptoethanol. 10 µL of each sample was run on SDS–PAGE and the gels were stained with Coomassie blue.

Visualizing the effect of Cry14Ab on the nematode intestine. BSA was labeled with Alexa Fluor 680 NHS Ester (Invitrogen). Cry14Ab was labeled with IRDye 680RD NHS Ester (LI-COR Biosciences). To test whether the label had affected the activity of Cry14Ab, labeled and unlabeled protein were tested in bioassays in a 2-fold titration with protein with concentrations ranging from 100 µg/mL to 0.01 µg/mL. About 600 C. elegans in 400 µL of S media containing 50 µg rifampin plus HB101 E. coli worm food were mixed with 100 µL of labeled protein to give a final protein concentration of 0.066 mg/mL. To detect ingestion the nematodes were incubated for 4 h, pelleted and rinsed with S media three times, anesthetized with 1% -Phenoxy-2-propanol, and imaged with white light or with a Cy5 filter set. To detect retention of Cry14Ab in the nematode intestine, the nematodes were incubated for 24 h, pelleted and rinsed with S media three times, incubated in S media for another 24 h, and imaged as before.

Production of transgenic soybean expressing Cry14Ab. For the expression of Cry14Ab in soybean two separate vectors were constructed for co-transformation, while a third vector was used to generate negative control plants (Supplementary Fig. 2). The first vector, pAXS547, contained the gene for Cry14Ab and the gene for a yellow fluorescent protein (YFP) marker. The second vector, pAXS207, contained the gene for the glyphosate selectable marker GRG23Ace5 (a modified 5-enolpyruvylshikimate-3-phosphate synthase), and the gene for phospho-serine hydrolase (PMI). The gene for Cry14Ab was re-coded from the native sequence to a soybean-optimized version. These two vectors were used to co-transform soybean cv. Jack using aerosol beaming of embryogenic callus46,47, and negative control plants were generated by transforming soybean with the third vector, pAXS209, which contained no selectable marker (GRG23Ace5) and the gene for green fluorescent protein 2 (GF2), and plants were again selected using the glyphosate resistance gene as a selectable marker during event regeneration. Negative control plants were generated by transforming soybean cv. Jack with the third vector, pAXS209, which contained no selectable marker (GRG23Ace5) and the gene for green fluorescent protein 2 (GF2), and plants were again selected using the glyphosate resistance gene as a selectable marker during event regeneration.

Cry14Ab detection by western blot. Leaf samples (0.5 cm leaf disks) were taken from full, newly emerged leaves and maintained at ~80 °C prior to processing. Samples were processed in 1.5 mL Eppendorf tubes by adding 200 µL of NP-40 lysis buffer to each sample and then freezing at −80 °C until required for analysis. The frozen samples were then subjected to protein extraction using a tissue homogenizer and the proteins were then subjected to SDS-PAGE. Bacterial cultures were grown in liquid medium to sporulation and then pelleted. The spore/crystal pellets were reconstituted with 20 mM CAPS pH 10.5, 10 mM beta-mercaptoethanol, 10% glycerol. For bioassays the protein was dialyzed to remove beta-mercaptoethanol and glycerol. For further purification, the protein was fractionated on a Superdex 200 column in the extraction buffer. The protein concentration was determined by running Coomassie-stained SDS–PAGE with known quantities of BSA for comparison.

To raise polyclonal antibodies against Cry14Ab, a version of the protein with a 6-his tag at the N-terminus was expressed in E. coli, and was extracted from a cell pellet in 50 mM Tris pH 8. After dialysis into 50 mM sodium phosphate pH 7, 300 mM NaCl, 10 mM imidazole, the protein was purified using cOBalt resin. The protein was purified to be >95% pure and was concentrated to ~4 mg/mL in 20 mM CAPS pH 10.5, 10 mM beta-mercaptoethanol, 10% glycerol with a large enough well to hold 1.7 mg of the protein. After Coomassie staining, the band corresponding to Cry14Ab was cut out and sent to Pacific Immunology for injection into rabbits. To make truncated Cry14Ab, the glycine at position 708 was mutated to alanine. The mutant protein was expressed in Bt as for wild-type protein and was cleaved with trypsin. The N-terminal fragment of the cleaved protein was purified by anion exchange chromatography and was of the expected size based on SDS–PAGE, and an aliquot was fluorescently labeled as for wild-type protein.

To detect retention of Cry14Ab in the nematode intestine, the nematodes were incubated for 24 h, pelleted and rinsed with S media three times, incubated in S media for another 24 h, and imaged as before.

Production of transgenic soybean expressing Cry14Ab. For the expression of Cry14Ab in soybean two separate vectors were constructed for co-transformation, while a third vector was used to generate negative control plants (Supplementary Fig. 2). The first vector, pAXS547, contained the gene for Cry14Ab and the gene for a yellow fluorescent protein (YFP) marker. The second vector, pAXS207, contained the gene for the glyphosate selectable marker GRG23Ace5 (a modified 5-enolpyruvylshikimate-3-phosphate synthase), and the gene for phospho-serine hydrolase (PMI). The gene for Cry14Ab was re-coded from the native sequence to a soybean-optimized version. These two vectors were used to co-transform soybean cv. Jack using aerosol beaming of embryogenic callus46,47, and negative control plants were generated by transforming soybean with the third vector, pAXS209, which contained no selectable marker (GRG23Ace5) and the gene for green fluorescent protein 2 (GF2), and plants were again selected using the glyphosate resistance gene as a selectable marker during event regeneration.

Cry14Ab detection by western blot. Leaf samples (0.5 cm leaf disks) were taken from full, newly emerged leaves and maintained at ~80 °C prior to processing. Samples were processed in 1.5 mL Eppendorf tubes by adding 200 µL of NP-40 lysis buffer to each sample and then freezing at −80 °C until required for analysis. The frozen samples were then subjected to protein extraction using a tissue homogenizer and the proteins were then subjected to SDS-PAGE. Bacterial cultures were grown in liquid medium to sporulation and then pelleted. The spore/crystal pellets were reconstituted with 20 mM CAPS pH 10.5, 10 mM beta-mercaptoethanol, 10% glycerol. For bioassays the protein was dialyzed to remove beta-mercaptoethanol and glycerol. For further purification, the protein was fractionated on a Superdex 200 column in the extraction buffer. The protein concentration was determined by running Coomassie-stained SDS–PAGE with known quantities of BSA for comparison.

To raise polyclonal antibodies against Cry14Ab, a version of the protein with a 6-his tag at the N-terminus was expressed in E. coli, and was extracted from a cell pellet in 50 mM Tris pH 8. After dialysis into 50 mM sodium phosphate pH 7, 300 mM NaCl, 10 mM imidazole, the protein was purified using cOBalt resin. The protein was purified to be >95% pure and was concentrated to ~4 mg/mL in 20 mM CAPS pH 10.5, 10 mM beta-mercaptoethanol, 10% glycerol with a large enough well to hold 1.7 mg of the protein. After Coomassie staining, the band corresponding to Cry14Ab was cut out and sent to Pacific Immunology for injection into rabbits. To make truncated Cry14Ab, the glycine at position 708 was mutated to alanine. The mutant protein was expressed in Bt as for wild-type protein and was cleaved with trypsin. The N-terminal fragment of the cleaved protein was purified by anion exchange chromatography and was of the expected size based on SDS–PAGE, and an aliquot was fluorescently labeled as for wild-type protein.
from the sand and the females were harvested and counted for each individual plant. Statistical analysis was done using R, version 3.5.2. Graphs were produced using Microsoft Excel, version 2008.

**Field trials.** A field plot experiment was planted in Webster County, IA in 2010 and 2011. Control and experimental event plants were planted in plots consisting of three rows 0.3 m long and spaced 76.2 cm apart. Plots were spatially isolated from each other with 1.8 m borders to prevent soybean roots from growing between plots. Plots were arranged in a randomized complete block design with four replications and were grown using normal cultural practices. For each entry, four replications of 24 plants each were planted. After PCR analysis, non-transgenic segregates were removed by hand. Controls in the trial were untransformed Jack. Jack transformed with the herbicide tolerance gene (gr23ace5) alone (also present in test plants), and a variety with no known resistance to any HG type of H. glycines (Lee’74 or Thorne). Soil samples collected from the middle of each plot were taken to estimate the population density of H. glycines. A soil sample consisted of six soil cores measuring 1.9 cm in diameter and taken to a depth of 15 cm. H. glycines eggs were extracted and counted from a representative 100 cc subsample of each six-core soil sample to determine the population density of H. glycines for each sample. One control plot and two Cry1Ab plots were lost after planting, and one Cry1Ab plot was lost after mid-season soil sampling. Statistical analysis was done using R, version 3.5.2. Graphs were produced using Microsoft Excel, version 2008.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this article is available as a Supplementary Information file. Cry1Ab is GenBank accession AGU13817.1 [https://www.ncbi.nlm.nih.gov/protein/533206134]. RefSeq, the NCBI Reference Sequence Database, is available at https://www.ncbi.nlm.nih.gov/refseq. The datasets and materials generated and analyzed during the current study are available from the corresponding author upon request, and a response can be expected within 2 months. The proteins, associated genes, and other aspects of the concept described in this article are protected under one or more patent filings and may also be subject to other intellectual property rights. BASF reserves the right to require a requester of such materials to enter into a non-disclosure agreement, a material transfer agreement, or other common type of agreement, in order to receive the materials. Further, some of the materials used to generate the data in this paper are highly regulated by government agencies and can only be shared with parties that meet all of BASF’s regulatory and stewardship requirements. The use of the materials will be limited to non-commercial research uses only. Source data are available in figshare [https://doi.org/10.6084/m9.figshar.14515587.v1]. Source data are provided with this paper.

Received: 19 November 2020; Accepted: 13 May 2021; Published online: 07 June 2021.

**References**

1. Nicol, J. M. et al. Current nematode threats to world agriculture. In Genomics and Molecular Genetics of Plant-Nematode Interactions (eds Jones, J. T., Gheysen, G. & Fenoll, C.) 21–44 (Springer, 2011).
2. Bandara, A. Y., Weerasooriya, D. K., Bradley, C. A., Allen, T. W. & Esker, P. D. Dissecting the economic impact of soybean diseases in the United States over two decades. PLoS ONE 15, e0231141 (2020).
3. Francisco, E., Câmara, G., Casarini, V. & Prochnow, L. Increasing soybean yields: Brazil’s challenges. Better Crops 98, 4 (2014).
4. Chen, S., Kurle, J., Malvick, D., Potter, B. & Orf, J. Soybean Cyst Nematode Management Guide [https://extension.umn.edu/soybean-pest-management/soybean-cyst-nematode-management-guide] (2018).
5. Iowa State University Extension and Outreach. Soybean Cyst Nematode Integrated Crop Management [https://crops.extension.iastate.edu/soybean/diseases_SCNtools.html] (2021).
6. Mitchum, M. G. Soybean resistance to the soybean cyst nematode Heterodera glycines: an update. Phytopathology 106, 1444–1450 (2016).
7. Roth, M. G. et al. Integrated management of important soybean pathogens of the United States in changing climate. J. Integr. Pest Manag. 11, 17 (2020).
8. Lee, T. G., Kumar, I., Diers, B. W. & Hudson, M. E. Evolution and selection of Rhgl, a copy-number variant nematode-resistance locus. Mol. Ecol. [https://doi.org/10.1111/mec.15118] (2015).
9. McCarville, M. T., Maret, C. C., Mullaney, M. P., Gebhart, G. D. & Tyllka, G. L. Increase in soybean cyst nematode virulence and reproduction on resistant soybean varieties in Iowa from 2001 to 2015 and the effects on soybean yields. Plant Health Prog. 18, 146–155 (2017).
10. Olson, S. An analysis of the biopesticide market now and where it is going. Outlooks Pest Manag. [https://doi.org/10.1564/v26_oct_04] (2015).
11. Sanahuja, G., Banalcar, R. M., Capell, T. & Christou, P. Bacillus thuringiensis: a century of research, development and commercial applications. Plant Biotechnol. J. 9, 283–300 (2011).
12. Hutchison, W. D. et al. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science [https://doi.org/10.1126/science.1190242] (2010).
13. Koch, M. S. et al. The food and environmental safety of Bt crops. Front. Plant Sci. 6, 283 (2015).
14. Ravari, S. B. & Moghaddam, E. M. Efficacy of Bacillus thuringiensis Cry14 toxin against root knot nematode, Meloidogyne javanica. Plant Prot. Sci. 51, 46–51 (2015).
15. Lu, X.-Q. et al. Expression of Cry5B protein from Bacillus thuringiensis in plant roots confers resistance to root-knot nematode. Biol. Control 47, 97–102 (2008).
16. Davis, E. L. & Tyllka, G. L. Soybean Cyst Nematode Disease [https://www.ncbi.nlm.nih.gov/protein/533206134]. RefSeq, the NCBI Reference Sequence Database, is available at https://www.ncbi.nlm.nih.gov/refseq. The datasets and materials generated and analyzed during the current study are available from the corresponding author upon request, and a response can be expected within 2 months. The proteins, associated genes, and other aspects of the concept described in this article are protected under one or more patent filings and may also be subject to other intellectual property rights. BASF reserves the right to require a requester of such materials to enter into a non-disclosure agreement, a material transfer agreement, or other common type of agreement, in order to receive the materials. Further, some of the materials used to generate the data in this paper are highly regulated by government agencies and can only be shared with parties that meet all of BASF’s regulatory and stewardship requirements. The use of the materials will be limited to non-commercial research uses only. Source data are available in figshare [https://doi.org/10.6084/m9.figshar.14515587.v1]. Source data are provided with this paper.

Received: 19 November 2020; Accepted: 13 May 2021; Published online: 07 June 2021.

**References**

1. Nicol, J. M. et al. Current nematode threats to world agriculture. In Genomics and Molecular Genetics of Plant-Nematode Interactions (eds Jones, J. T., Gheysen, G. & Fenoll, C.) 21–44 (Springer, 2011).
2. Bandara, A. Y., Weerasooriya, D. K., Bradley, C. A., Allen, T. W. & Esker, P. D. Dissecting the economic impact of soybean diseases in the United States over two decades. PLoS ONE 15, e0231141 (2020).
3. Francisco, E., Câmara, G., Casarini, V. & Prochnow, L. Increasing soybean yields: Brazil’s challenges. Better Crops 98, 4 (2014).
4. Chen, S., Kurle, J., Malvick, D., Potter, B. & Orf, J. Soybean Cyst Nematode Management Guide [https://extension.umn.edu/soybean-pest-management/soybean-cyst-nematode-management-guide] (2018).
5. Iowa State University Extension and Outreach. Soybean Cyst Nematode Integrated Crop Management [https://crops.extension.iastate.edu/soybean/diseases_SCNtools.html] (2021).
6. Mitchum, M. G. Soybean resistance to the soybean cyst nematode Heterodera glycines: an update. Phytopathology 106, 1444–1450 (2016).
7. Roth, M. G. et al. Integrated management of important soybean pathogens of the United States in changing climate. J. Integr. Pest Manag. 11, 17 (2020).
8. Lee, T. G., Kumar, I., Diers, B. W. & Hudson, M. E. Evolution and selection of Rhgl, a copy-number variant nematode-resistance locus. Mol. Ecol. [https://doi.org/10.1111/mec.15118] (2015).
9. McCarville, M. T., Maret, C. C., Mullaney, M. P., Gebhart, G. D. & Tyllka, G. L. Increase in soybean cyst nematode virulence and reproduction on resistant soybean varieties in Iowa from 2001 to 2015 and the effects on soybean yields. Plant Health Prog. 18, 146–155 (2017).
Acknowledgements
We thank Jun Cao for performing the plant transformations, Jayme Williams for preparing the truncated version of Cry14Ab, Jeanett Perez-Lesher for preparing vector illustrations, and Cheryl Peters for technical support.

Author contributions
T.W.K., N.B.D., and J.D. contributed to the conception, design, and interpretation of the study; T.W.K., L.C.S., K.S., J.Z., and J.D. performed the experiments; T.W.K., M.T.M., L.C.S., K.S., J.Z., and J.D. contributed sections to the manuscript; T.W.K. and J.D. wrote and compiled the final manuscript. All the authors reviewed the manuscript.

Competing interests
At the time this work was carried out the authors were employees of BASF, a for-profit company. BASF holds or has applied for patents and/or other intellectual property rights on the proteins, associated genes, and other aspects of the concept described in this article.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-23743-3.

Correspondence and requests for materials should be addressed to T.W.K.

Peer review information Nature Communications thanks Neil Crickmore and other, anonymous, reviewers for their contributions to the peer review of this work. Peer review reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access
This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021