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Expression of Cry5B protein from *Bacillus thuringiensis* in plant roots confers resistance to root-knot nematode

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ABSTRACT

Plant-parasitic nematodes are major pests of agricultural crops, and it is becoming increasing urgent to find new methods to control them. *Bacillus thuringiensis* (Bt) has been used for decades as a biological control agent against insect pests and occurs as a natural pathogen of nematodes, but its potential to control nematode pests has been largely ignored. To test whether Bt can be used for control against nematodes, we decided to express the active component of Bt, namely an ingestible crystal (Cry) protein, inside plant roots where the nematodes feed. Truncation studies here demonstrate that the first 2094 nucleotides of the nematicidal Cry protein, Cry5B, are sufficient for toxicity against a free-living nematode. Based on these results, a plant codon-modified *cry5B* gene was synthesized and transformed into tomato hairy roots. Cry5B expression in hairy roots was assessed at the mRNA level and quantified at the protein level. Characterization and quantification of root-knot nematode infections on control versus Cry5B-expressing roots indicate that Cry5B intoxicates the parasitic nematode as judged by inhibition of the nematode to establish or maintain root-knot sites and as judged by a nearly 3-fold reduction in progeny production. We demonstrate for the first time that a three-domain Cry protein can provide protection against a plant-endoparasitic nematode and that Cry proteins as a group have excellent potential as control agents of plant-parasitic nematodes.

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1. Introduction

Plant-parasitic nematodes (PPNs) are major yield limiting factors in many crops including potato, sugar beet, soybean, tomato, and others (Jung and Wyss, 1999; Williamson and Gleason, 2003). They are very difficult pests to control since many destructive PPNs are endoparasites. They live underground and spend most of their lives in the roots, which can protect them even from chemical treatments. For instance, juveniles of the root-knot nematode, *Meloidogyne incognita* (Kofoid & White) Chitwood, move intercellularly after penetrating the root and establish a permanent feeding site in the differentiation zone of the root (Wyss et al., 1992). Although chemicals can be used for PPN control, e.g., methyl bromide, these compounds tend to be highly toxic to people and the environment. Expressing environmentally friendly nematicidal proteins inside the root is a potentially excellent alternative strategy for control of PPNs (Urwin et al., 2001).

Our lab has pioneered work on crystal (Cry) proteins made by *Bacillus thuringiensis* (Bt) Berliner that target nematodes (Crickmore, 2005; Marroquin et al., 2000; Wei et al., 2003). In particular, we have focused on Cry5B, a three-domain Bt Cry protein, predicted to have the same structure as insecticidal proteins used in transgenic plants (Barrows et al., 2007; Griffitts et al., 2005, 2001; Huffman et al., 2004; Marroquin et al., 2000). We have shown that Cry5B uses an invertebrate-specific glycolipid as its receptor to kill nematodes, including animal-parasitic nematodes *in vitro* and *in vivo* (Barrows et al., 2007; Cappello et al., 2006; Griffitts et al., 2005). Bt spore-crystal lysates and the active ingredient of the lysates, namely Cry proteins, have been used as biological control agents against insect pests with great success for decades (Glare and O'Callaghan, 2000; Whalon and Wingerd, 2003). Attractive features of these proteins include their long-term effectiveness at controlling insect pests in parallel with their demonstrated non-toxicity towards vertebrates. The use of transgenic crops expressing insecticidal Cry proteins has resulted in excellent control of insect pests with a concomitant and significant decrease in the use of chemical insecticides, resulting in improvements in crop yield and in significant environmental and farmer health benefits (Huang et al., 2005; Qaim and Zilberman, 2003). However, the question of whether Cry proteins can be used to control PPNs has largely been ignored.

Here we test for the first time whether or not the three-domain Cry protein, Cry5B, made by Bt is toxic to a PPN when expressed in a plant. We produce transgenic roots that express Cry5B and test

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the ability of these roots to provide control of *M. incognita*. We find that Cry5B expression significantly reduces the number of galls and also reduces the amount of progeny produced by nearly 3-fold.

2. Materials and methods

2.1. Testing Cry5B truncated constructs for toxicity against Caenorhabditis elegans Maupas

Truncated Cry5B constructs were amplified from full-length Cry5B in *Escherichia coli* (Migula) (Wei et al., 2003) with different primer combinations (forward primer: TGTCAC**CATATG**GCAACAAT TAATGAGTTG; nt 2094 reverse primer CCG**CTCGAG**TTATTGGATTTT TGGAACAAACTC; nt 2142 reverse primer CCG**CTCGAG**TTAAGGGTT ATTACAATCACAG). After digestion with NdeI (5′ end) and XhoI (3′ end), DNA fragments were then cloned into pET-20b(+) vector (Novagen, Madison, WI). These constructs were transformed into the *E. coli* strain Rosetta pLysS (Novagen), where expression is stringently controlled. Full-length toxin (to nucleotide 3735) cloned in pQE9 vector was used from a previously generated construct (Wei et al., 2003) and transformed into Rosetta pLysS as positive control. The transformed cells were recovered by shaking at 37 °C for 1 h. The recovered cells were diluted 6 times with Luria–Bertani broth medium containing 50 mg/L carbenicillin. Three hundred microliters of the overnight culture was spread on a *C. elegans* NG plate containing 0.25 mM IPTG and 30 mg/L carbenicillin, incubated at room temperature overnight, and then inoculated with five first-stage wild-type juveniles (J_1) *C. elegans* (N2) per plate onto four plates, which were placed at 20 °C. The assay was independently repeated. The toxicity was determined by observation of the overall health of the worms (motility, coloration, size) at 40 and 64 h. Photos shown were taken at 64 h.

2.2. Gene synthesis and vector construction

Codon-modified *cry5B* gene was designed as described in the text and synthesized by assembling the entire gene *de novo* from 70 to 90-mer oligonucleotides (EtonBio, San Diego, CA) and inserting into the pBluescript KS (+) vector (Stratagene, La Jolla, CA). The synthesized genes next were subcloned into the plant-expression binary vector, pBIN-JIT (Ferrandiz et al., 2000), using SalI and BamHI cloning sites introduced at the 5′ and 3′ ends of the gene. The entire sequence of the codon-modified gene has been deposited with GenBank Accession No. EU822809.

2.3. Plant transformation

Tomato (*Lycopersicon esculentum* Mill. var. Rutgers select) hairy roots were generated by transforming cotyledons with *Agrobacterium rhizogenes* (Riker et al.) strain R1000, containing either empty vector or vector containing *cry5B*, as described (Li et al., 2007). Over 20 vector-transformed lines and over 50 *cry5B*-transformed hairy root lines were isolated. The two control lines and three Cry5B-expressing lines were selected based on their health, the level of Cry5B expressed, and the fact that they all showed similar, well matched morphology. Root growth assays were carried out as described (Li et al., 2007).

2.4. Detection of protein expression in tomato hairy roots

About 100 mg of fresh hairy root tissue was ground in liquid nitrogen, to which was then added 200 µl of extraction buffer 100 mM Tris–HCl pH 7.5, 100 mM NaCl, 25 mM DTT, 10 mM EDTA, 5 mM benzamidine–HCl, 5 mM PMSF, 50 µM phenanthroline, 1.5 µM aprotinin, 15 µM pepstatin A, 20 µM leupeptin, and 1× proteinase inhibitor cocktail P9599 (Sigma–Aldrich, St. Louis, MO). Following 10 min centrifugation at 12,000*g* at 4 °C, the total soluble protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA, Sigma A7906) as the protein standard. The protein samples were separated on 8% SDS polyacrylamide gels and immunoblotted onto nitrocellulose membranes.

Polyclonal rabbit-anti-5B antibodies were developed from full-length Cry5B protein (Griffitts et al., 2001) injected into rabbits (Covance, Denver, PA). Polyclonal rabbit-anti-5B antibody (used at 1:1000) was used to probe blots containing protein extracts from hairy roots and known amounts of purified, trypsin-activated Cry5B protein as a control. The 79 kDa *E. coli* produced Cry5B is not available for these analyses since it is produced at such low levels in *E. coli*. For use in Western blotting experiments, Cry5B antibody was affinity-purified against 65 kDa trypsin-activated protein (Griffitts et al., 2001), which is mostly contained within the truncated 79 kDa protein expressed in plants. Thus, even though the size of the loading control is different from that of root expressed Cry5B, the binding capacity of antibody is likely similar for both proteins. Western blotting was carried out as described (Li et al., 2007). Cry protein expression was detected using horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA, 111-035-003) and a chemiluminescence detection kit (Super Signal West Pico, Pierce, Rockford, IL).

2.5. RT-PCR and Northern blot analysis

Total RNA was isolated from fresh hairy roots using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD). For RT-PCR analysis, the first strand of cDNA was synthesized by M-MLV reverse transcriptase (Promega, Madison, WI) with oligo d(T) linked to an adapter sequence, 5′-GACGATGAGTCTACCAGGATCCTTTTTTTTTTTTTTTTT T. This cDNA was then PCR amplified using the adapter sequence without the oligo d(T) tail (GACGATGAGTCTACCAGGATCC) as the 3′ end primer and the 5′ end of *cry5B* as the 5′ end primer (ATGACAAATCCTACTATTCTTTACC). PCR conditions were: 94 °C 3 min (94 °C 30 s, 55 °C 45 s, 72 °C 2 min 30 s) × 30 cycles, followed by 72 °C 7 min. After amplification, the PCR product was sequenced. For Northern blot analyses, 10 µg of total RNA was electrophoresed in a 1.2% formaldehyde–agarose gel as described in the RNeasy Plant Mini Kit instructions. RNA transfer, probe labeling, hybridization, and washing were carried out as described (Sambrook et al., 1989).

2.6. M. incognita maintenance and bioassays

Meloidogyne incognita populations were maintained on tomato plants as described (Li et al., 2007). To set up bioassays of *M. incognita* infection, second-stage juvenile (J2) worms were hatched from sterilized eggs and used for infection of hairy roots as described (Li et al., 2007). At least three independent sets of bioassays were carried out for all lines, and a minimum of 19 plates/line were assayed.

2.7. Statistical analyses

Each plate was taken as an individual data point for input into statistical analyses. The two control lines were compared using Student's *t*-test and were found to be statistically equivalent for all three outcomes (total galls, total egg masses, total eggs; $P = 0.246, 0.957, 0.533$) and for the ratios of all three outcomes. We therefore combined the two control groups for all subsequent analyses. For comparison of control group against each of the Cry5B lines, ANOVA and pairwise comparisons with LSD adjust-

ment were performed. The significance level was set at 0.05. All data analysis was performed using SPSS, ver 13.0 (SPSS, Chicago, IL). The number of total galls is significantly different among groups ($F = 12.178$; degrees of freedom (df) = 3, 97; $p < 0.001$). The number of total egg masses is significantly different among groups ($F = 6.038$; df = 3, 97; $p < 0.001$). The number of total masses is significantly different among groups ($F = 6.117$; df = 3, 97; $p < 0.001$).

3. Results

3.1. Structure–function analysis of Cry5B

Cry5B is a three-domain crystal protein predicted to fold into the same three-dimensional structure as the three-domain Cry proteins used in transgenic crops, e.g., Cry1Aab (de Maagd et al., 2001; Marroquin et al., 2000; Schnepf et al., 1998). Three-domain crystal proteins are often synthesized as large protoxins that contain smaller active toxin cores that end just carboxy-terminal to block 5, which is well conserved in Cry5B. Expressing short, active toxins in plants are highly desirable since it is significantly more difficult to get larger proteins to express well in plant tissues. Indeed, Bt Cry proteins engineered to be expressed in transgenic plants are truncated close to the end of block 5 (Koziel et al., 1993).

To determine whether Cry5B could also be truncated to a shorter form, we cloned *cry5B* genes with different 5′ and 3′ truncations into an *E. coli* high expression vector and transformed these into the *E. coli* strain JM103, the strain we used for expressing full-length Cry5B and other Cry proteins (Wei et al., 2003). However, we could not recover truncated *cry5B* constructs with the correct DNA sequence this way, suggesting that truncated Cry5B might be toxic to *E. coli* at high levels of expression. We then cloned truncated *cry5B* genes into the pET-20b(+) expression vector and transformed these into the *E. coli* strain Rosetta pLysS, where expression is stringently controlled. Truncated Cry5B expressed under these conditions is not abundant enough to be visualized on Coomassie gels but could be detected by Western blots (data not shown). We found that C-terminal truncations that retain the first 2094 nucleotides (encoding five amino acid behind DRIEF, which is known as block 5 and is at the end of the active toxin domain of three-domain Cry proteins (Schnepf et al., 1998)) are still highly toxic against *C. elegans* (Fig. 1). At the other end, N-terminal deletion of as little as 63 nucleotides weakens the toxicity dramatically (data not shown). Based on these data, we opted to construct, for expression in plants, a *cry5B* gene starting at nucleotide 1 and ending at nucleotide 2094.

3.2. Transformation and expression of Cry5B in plant roots

Wild-type bacterial genes encoding Cry proteins are poorly expressed in transgenic plants due to the fact that bacterial Cry genes often contain sequences interpreted by the plant as polyadenylation sites, introns, or signals for mRNA destabilization. Thus, we synthesized a 2094 nucleotide "plant-friendly" version of *cry5B* (with the translation initiation sequence AAA**ATG**GC and a stop codon at the end) based on the codon usage of *Arabidopsis* genes along with a slight increase in the frequency of NNG/NNC codons to increase the overall GC content. Furthermore, any occurrences of ATTTA (which destabilizes mRNA in plants), AATGAA, AATATT, GATAAA, AATTAA, AATAAA, and AATAAT (which are potential polyA signal sequences), and potential splice sites (see http://www.cbs.dtu.dk/services/NetPGene/ and http://genes.mit.edu/GENSCAN.html) were excluded. The G–C content of the *cry5B* gene was increased from 32% (bacterial gene) to 48% (plant construct). The resulting protein is predicted to express from amino acid number one to five amino acids after box 5 (DRIEF). The codon-modified *cry5B* gene was cloned into the pBIN-JIT vector driven by tandem cauliflower mosaic virus (CaMV) 35S promoters and introduced via *Agrobacterium rhizogenes* into tomato cotyledons to generate transgenic hairy roots.

To make sure that the Cry5B transgenic roots express the correct transcripts, we analyzed RNA from two hairy root lines that were found to express Cry5B protein (see below). We extracted total RNA from hairy roots generated from each construct and carried out both RT-PCR and Northern blot analyses. We observed an RT-PCR product at the predicted correct size of transcript, as well as some smaller sized PCR products (Fig. 2A). Sequencing of RT-PCR products indicated that *cry5B* transcripts in transgenic roots are complete and correct at the nucleotide level. We did not observe aberrant transcripts in Northern blot analysis (Fig. 2B). Thus, the smaller sized PCR products from the RT-PCR analyses might arise from non-specific amplification. Some of these bands also appeared in vector control root lines and might arise from the adapter sequence used for the PCR reaction.

To quantify Cry5B expression levels and to determine which lines to select for nematode tests, total soluble protein was extracted from 36 tomato hairy roots lines and subjected to Western blotting probed with anti-Cry5B antibody. On the same blot, known quantities of trypsin-activated Cry5B were also loaded. The results from five Cry5B-expressing lines are shown (Fig. 2C). Based on comparison to loading controls, the expression level of Cry5B in the highest expressing lines is estimated to be 0.1% of total soluble protein.

3.3. Control of M. incognita by Cry5B expression in roots

To test the effects of Cry5B expression on PPN infection and development, *M. incognita* infection assays were performed as described (Li et al., 2007). A total of five transgenic root lines were used for our study. Two independently generated lines from transformation with empty pBIN-JIT vector were used as control, designated as 70S-7 and 70S-14. Three cry5B lines (5B-15, 5B-31, and 5B-32) expressing various levels of Cry5B protein were used to test

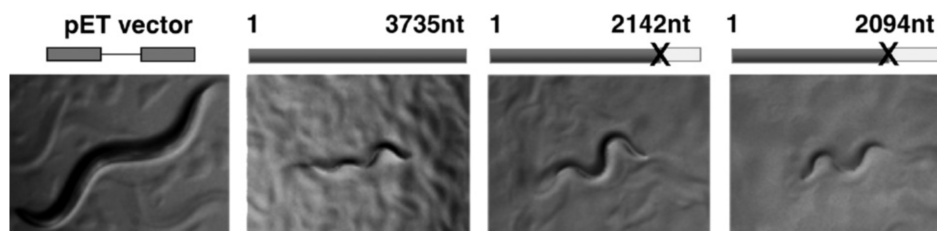

Fig. 1. Toxicity of Cry5B truncations on *Caenorhabditis elegans*. Above each panel are indicated the construct being expressed in *Escherichia coli* (the narrow line between bars in left panel signifies empty vector; "X" marks the location of the truncations in Cry5B). In each panel are shown a typical *C. elegans* animal grown on that *E. coli*. Animals grown on empty vector (left panel) are relatively large and healthy. Animals grown on full-length Cry5B (second panel) or Cry5B truncated up to nucleotide 2094 (third and fourth panels) are small and unhealthy, indicating intoxication.

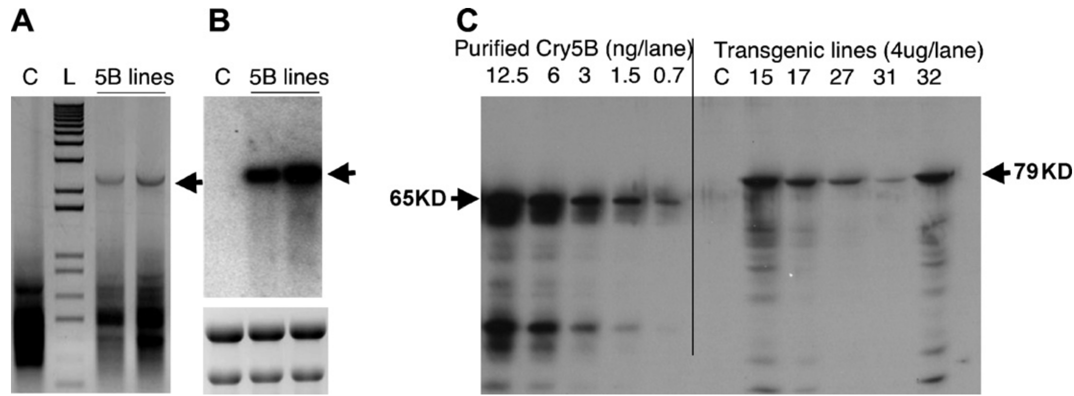

Fig. 2. Characterization of Cry5B expression in tomato roots. Total RNA was isolated from two Cry5B-expressing lines. (A) Reverse transcription-PCR analyses. RT RNA was PCR amplified with 3′ (adapter) and 5′ (*cry5B*-specific) primers. Sequencing of these RT-PCR products demonstrated that the *cry5B* transcripts contain correct coding and terminator sequences. C = RNA from control empty vector line, L = 1 kb ladder. Arrows here and in (B) indicate full-length Cry5B bands. (B) Northern blot was carried out with a total of 10 μg total RNA and probed with P^{32}-labeled full-length *cry5B*. Bottom panel: intensities of ribosomal RNA bands were similar from control and two transgenic lines after gel electrophoresis, confirming equal loading. (C) Cry5B expression in tomato hairy roots was quantified with Western blot probed with affinity-purified anti-Cry5B antibody. Left lanes: Known amounts of Cry5B protein purified from *Bacillus thuringiensis*. Right lanes: total soluble protein from hairy root lines (4 μg protein/lane). Among these lines, 5B-15, 5B-31, and -32 were later used for intoxication assay of *M. incognita*. C = protein from empty vector-transformed root line.

the effect of Cry5B on *M. incognita*. Cry5B-15 and -32 expressed the highest level of Cry5B, ~0.1% of total soluble protein (Fig. 2C). Cry5B-31 expressed a lower level of Cry5B, roughly 4–5 times less than the other two lines. The root lines grew similarly (Fig. 3), except for 5B-31, which grew somewhat faster than the other lines. Otherwise there are no major differences amongst them, and the lines were morphologically well matched.

These five lines were inoculated with freshly hatched *M. incognita* J2s and then scored at 45 days post-infection for three different measures of infections: total number of galls formed, total number of egg masses formed, and total number of eggs produced (progeny production). The experiment was independently repeated at least 2 more times, and the data averaged and normalized relative to the empty vector-transformed roots.

We found the total number of galls from all three Cry5B lines lower than that on control roots. The number of galls on 5B-15, 5B-31, and 5B-32 roots, respectively, decreased to 65%, 75%, and 56% of that of empty vector roots (Fig. 4A).

Even larger reductions could be seen on the ability of the nematodes to reproduce. In lines 5B-15, -31, and -32, the number of egg masses decreased to 49%, 48%, and 56% relative to that of control roots (Fig. 4B). The total number of eggs produced from 5B-15, -31, and -32 lines was, respectively, 36%, 50%, and 54% relative to that of control roots (Fig. 4C). For all three outcomes and all three Cry5B-expressing root lines, the reductions seen were statistically significant ($p < 0.05$) relative to control roots. Thus, Cry5B can negatively impact the ability of root-knot nematode to reproduce on tomato roots.

4. Discussion

Here we demonstrate for the first time that a three-domain Bt crystal protein, Cry5B, can provide control of an endoparasitic nematode, *M. incognita*, when expressed in tomato roots. Bt, a staple of insect biological control, is a natural pathogen of nematodes (Rae et al., 2008; Schulenburg and Muller, 2004; Wei et al., 2003). Since endoparasitic PPNs feed only when inside the root and since the main virulence factors of Bt are its ingestible Cry proteins, testing the ability of Bt to control these parasites seems best achieved by expressing a Cry protein directly in the roots.

Expression of Cry5B in roots results in a decrease in the number of galls seen 45 days after infection and in up to a nearly 3-fold reduction in the ability of the parasite to reproduce in roots. Since Cry5B is predicted to be a three-domain Bt Cry protein, it is likely to have the same excellent non-toxicity profile towards vertebrates as other three-domain Cry proteins (Betz et al., 2000). Indeed, the receptor for Cry5B in the nematode *C. elegans* is an invertebrate-specific glycolipid, indicating that this protein should have a high

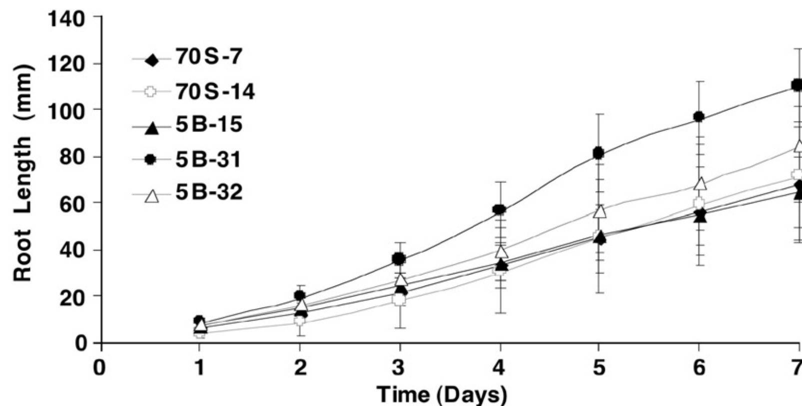

Fig. 3. Characterization of root growth rate. The lengths of transformed roots starting from 2 cm-long root tips were measured on 6 consecutive days. 70S-7 and 70S-14 are control lines. 5B-15, 5B-31, and 5B-32 are Cry5B-expressing lines.

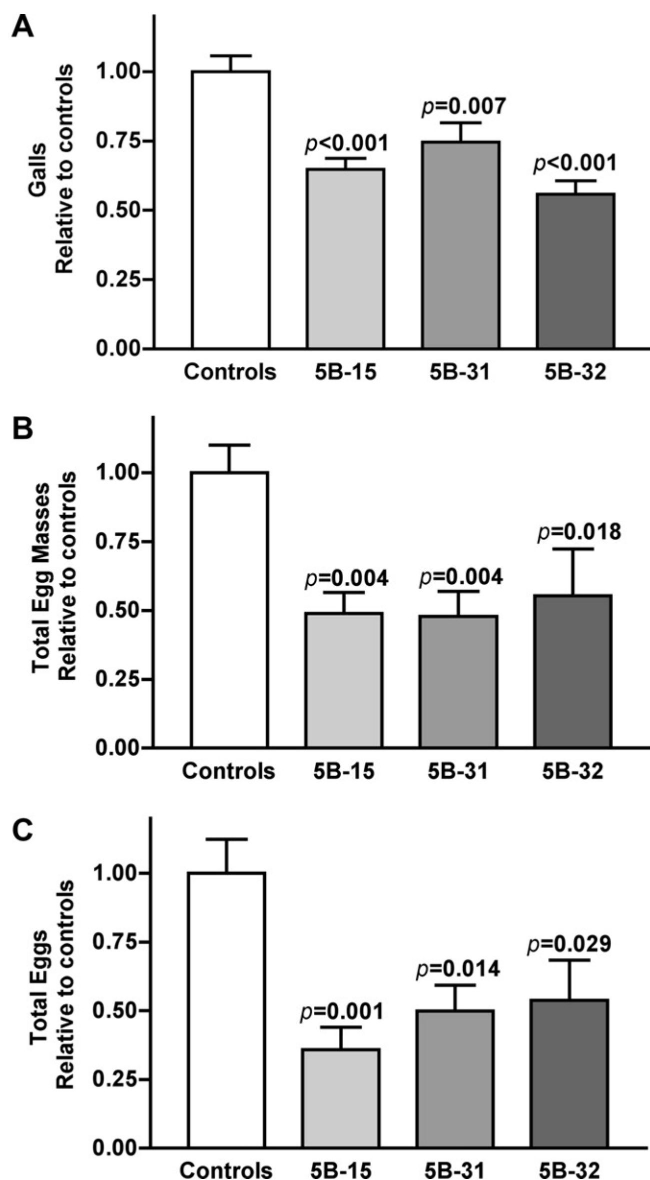

Fig. 4. Effects of Cry5B on *Meloidogyne incognita* infections as measured by (A) number of galls formed normalized to control roots; (B) egg mass production normalized to control roots and (C) total egg production normalized to control roots. The *p*-values relative to control roots given above each bar. Error bars represent the standard deviation.

degree of safety towards vertebrates (Barrows et al., 2007; Griffitts et al., 2005). Although it is difficult to isolate sufficient quantities of glycolipid material from dissected *M. incognita* adults to get more than a weak signal, our observations suggest that *M. incognita* adults also express Cry5B-binding glycolipids receptors (X.L. and R.V.A., unpublished data).

Cry5B reduces all parameters of infection measured—the number of galls seen 45 days after infection, the number of egg masses produced, and the number of eggs produced. The fact that the number of galls is reduced suggests that Cry5B is able to intoxicate the nematodes early in their feeding process thereby preventing the development of large galls and/or that Cry5B intoxication of nematodes causes galls to recede over time. Cry5B may be predominantly affecting early stages of infection and nematode development is supported by further statistical analyses. The number of total eggs produced per gall was reduced in all three lines, with the 5B-15 and 5B-31 lines showing the greatest reductions at 48% and 55% relative to control roots, respectively ($p = 0.020$ and 0.059, respectively, based on ANOVA analyses). Although the *p*-value in the reduction in the 5B-31 lines is slightly larger than the accepted cut-off of $p = 0.05$, we note that the 60% reduction in the number of egg masses produced per galls in this line is significant ($p = 0.036$). However, no statistically significant differences in the eggs per egg mass were seen relative to controls in any of the lines. Taken together, these data suggest that the most significant effect of Cry5B occurs on larva or early adults, rather than on the larger, egg-bearing adults (females). Consistent with this, we find that earlier larval stages of free-living nematodes are more susceptible to intoxicating effects of Cry proteins than later larval/adult stages (Wei et al., 2003).

An unexpected aspect of our data is that the 5B-31 line shows similar toxic effects as the two other Cry5B lines, although it expresses a lower amount of Cry5B. One explanation may lie with the availability of active Cry5B in the roots. It is possible that 5B-31 roots contain similar levels of bioavailable Cry5B as 5B-15 and 5B-32 lines to the plant parasite that is not reflected in the amounts of total soluble Cry5B detected in our quantitation experiments.

These data now bring to a total of two the number of Cry proteins that, when expressed in plants, can achieve control of a PPN. Expression of the non-canonical, non-three-domain Cry protein, Cry6A, in plants can significantly reduce the brood size of *M. incognita* in plants (Li et al., 2007). The results reported here extend the previous results to a canonical three-domain Cry protein, i.e., Cry5B. Having multiple Cry proteins to target nematode pests is highly desirable since one might be more effective against a specific PPN than another, as is typical of insecticidal Cry proteins (Hernandez-Martinez et al., 2008; Karim et al., 2000; Monnerat et al., 2006). Furthermore, animals resistant to Cry5B are not qualitatively resistant to Cry6A (Marroquin et al., 2000). This opens up the intriguing possibility of combining Cry5B and Cry6A expression into the same plant. Such a stacked plant, since it expresses two Cry with different resistance profiles, is likely to make it much more difficult for the animals to efficiently develop resistance to the transgenic plant, as has been demonstrated for stacking of insecticidal Cry proteins (Zhao et al., 2003).

The reductions seen in gall numbers and in progeny production by Cry5B in transgenic roots are significant, and this level of control could play a significant role as part of an integrated pest management program for PPNs. Given the long and distinguished track record of Bt Cry proteins for vertebrate safety and for effective pest control, our results demonstrate that nematicidal Bt Cry proteins have excellent potential to participate in the next generation of strategies for the control of PPNs.

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