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Effects of insecticidal crystal proteins (Cry proteins) produced by genetically modified maize (Bt maize) on the nematode *Caenorhabditis elegans*

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ABSTRACT

The genetically modified maize MON89034 × MON88017 expresses different crystal (Cry) proteins with pesticidal activity against the European corn borer (Cry1.105; Cry2Ab2) and the Western corn root worm (Cry3Bb1). Non-target organisms, such as soil nematodes, might be exposed to the Cry proteins that enter the soil in course of crop growing. Therefore, the risk of those proteins for nematodes was assessed by testing their toxic effects on *Caenorhabditis elegans*. All three insecticidal Cry proteins showed dose-dependent inhibitory effects on *C. elegans* reproduction (EC50: 0.12–0.38 μmol L^{-1}), however, at concentrations that were far above the expected soil concentrations. Moreover, a reduced toxicity was observed when Cry proteins were added jointly. A *C. elegans* mutant strain deficient for receptors for the nematicidal Cry5B was also resistant against Cry1.105 and Cry2Ab2, suggesting that these Cry proteins bound to the same or similar receptors as nematicidal Cry proteins and thereby affect the reproduction of *C. elegans*.

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1. Introduction

The invertebrate-specific pathogen *Bacillus thuringiensis* (Bt) produces pore-forming crystal (Cry) proteins with lethal effects on insects and has therefore been extensively used as a bio-rational pesticide to control crop-damaging insects (Schnepf et al., 1998). This pesticidal effect has also been deployed in genetic engineering to produce pest-resistant plants by transferring *cry* genes from Bt to the plant genome. For example, Bt-maize has been genetically modified to produce Cry1 and Cry3 proteins specifically acting against caterpillars (Lepidoptera) and beetles (Coleoptera), respectively. However, specific families of Cry proteins can also be toxic to organism groups other than insects, such as nematodes, mites, and protozoans (Feitelson et al., 1992; Wei et al., 2003). Cry proteins exert their effects on nematodes by a similar mode of action as insects, i.e., by binding to glycolipid receptors on intestinal cells and damaging the gut by cytolytic pore formation (Marroquin et al., 2000; Griffitts et al., 2003; Wei et al., 2003). Therefore, to study effects of Cry proteins on insects, the nematode *Caenorhabditis elegans* is a suitable model organism (Crickmore, 2005). Indeed, nematodes were shown to be susceptible to insecticidal Cry proteins such as Cry1Ab (Höss et al., 2008) and Cry3Bb1 (Höss et al., 2011), although, at concentrations that are much higher than expected field concentrations. Furthermore, a dose-dependent inhibition of *C. elegans* reproduction was demonstrated in aqueous solutions of Cry1Ab and Cry3Bb1 (Höss et al., 2008, 2011), together with the up-regulation of genes (Höss et al., 2011) known to be involved in the defense mechanism against nematicidal Cry proteins (Huffman et al., 2004a).

The maize line MON89034 × MON88017 contains stacked multiple Bt-genes for resistance against the European corn borer (*Ostrinia nubilalis*; Lepidoptera) and the Western corn rootworm

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(*Diabrotica virgifera virgifera*; Coleoptera). These plants produce three different Cry proteins, one that is coleopteran-specific (Cry3Bb1) and two that specifically target Lepidoptera (Cry1.105; Cry2Ab2). During the cultivation of MON89034 × MON88017, these Cry proteins may, as shown for Mon810 and Mon88017, enter the soil through root or plant residues (Zwahlen et al., 2003; Zurbrügg et al., 2010) or through feces deposited by animals that have fed on Bt-maize material (Weber and Nentwig, 2006; de Vaufleury et al., 2007). In fact, after the cultivation of the genetically modified maize lines Mon810 and Mon88017, Cry1Ab and Cry3Bb1 could be detected in bulk and rhizosphere soil, respectively, albeit in relatively low concentrations (peak concentrations in rhizosphere soil: Cry1Ab: <10 ng g^{-1} dry soil; Cry3Bb1: <1 mg g^{-1} dry soil; Baumgarte and Tebbe, 2005; Miethling-Graff et al., 2010). Thus, in the soil of Bt-maize fields, free-living nematodes are likely to be exposed to Cry proteins.

Nematodes are important components of the soil food web and, as such, should be considered in the risk assessment of genetically modified plants (GMP) for soil ecosystems. As a first step, the toxicity of the Cry proteins that represent the major source of risk emanating from Bt-maize should be known. Toxicity thresholds should be set for individual Cry proteins and, if exposure to more than one Cry protein is expected, for mixtures of Cry proteins. These toxicity thresholds can then be compared to predicted or measured concentrations in the field to forecast the risk posed by the presence of Cry proteins in agricultural soil.

To assess the risk of the maize MON89034 × MON88017 for soil nematodes, it is important to have detailed information about the toxicity of the Cry proteins that are produced by the plants to protect themselves against insect pests. Thus, the aim of the present study was to assess the toxicity of three different purified Cry proteins with pesticidal activity against *O. nubilalis* (Cry1.105; Cry2Ab2) and *D. v. virgifera* (Cry3Bb1) on the reproduction of the nematode *C. elegans*, after its exposure to the three proteins individually and as a mixture. To elucidate the mode of action of the insecticidal Cry proteins in nematodes, the expression of selected genes known to be involved in the defense mechanism against nematicidal Cry proteins was examined. In addition, a mutant strain of *C. elegans* deficient in receptors for nematicidal Cry proteins was compared with the wild type with respect to the effects of the insecticidal proteins. With purified nematicidal Cry5B protein serving as the positive control, experiments were designed to test the following hypotheses: (1) The nematicidal Cry5B protein is significantly more toxic to *C. elegans* than the insecticidal Cry proteins. (2) The mixture of the different insecticidal Cry proteins has a concentration-additive effect on *C. elegans*. (3) "Defense genes" are triggered by both the nematicidal and the insecticidal Cry proteins. (4) The absence of Cry protein receptors reduces the toxicity of nematicidal and insecticidal Cry proteins.

2. Materials and methods

2.1. Preparation of purified Cry protein solutions

Cry3Bb1 was expressed in *Escherichia coli* BL21 DE3 as described in Höss et al. (2011). The open reading frames of *cry1.105* and *cry2Ab2* were PCR-amplified using isolated DNA from the leaves of MON89034 × MON88017 as template, iProof High-Fidelity DNA polymerase (Biorad), and sequence-specific oligonucleotides deduced from the published sequences of the gene constructs of MON89034 and MON88017. The PCR fragments were cloned in *E. coli* DH5α into the pGEM-T Easy cloning vector (Invitrogen) and sequenced by Sanger sequencing. After the correct nucleotide sequence was corroborated, the *cry1.105* and *cry2Ab2* fragments were subcloned into the pET28b expression vector (Novagen) and expressed in *E. coli* BL21-DE3. Cry1.105 and Cry2Ab2 expression was induced by incubation of the recombinant *E. coli* strains with 0.05 mM IPTG at 20 °C for 16 h. These conditions resulted in the formation of non-soluble inclusion bodies for Cry1.105 and a 40% soluble protein for Cry2Ab2. For a detailed description of the protein purification, see Höss et al. (2011). The purity and biological activity of the expressed proteins were determined by SDS gel electrophoresis and in bioassays using neonate larvae of *Leptinotarsa decemlineata* (Cry3Bb1) according to Meissle and Romeis (2009) and *Ostrinia nubilalis* (Cry1.105 and Cry2Ab2) as described in Nguyen and Jehle (2009).

Sucrose-gradient purified Cry5B was purchased from the University of California San Diego (Raffi Aroian Lab) and stored at −80 °C until needed. Cry5B was dissolved in double-distilled water to a concentration of 2.3 μg μL^{-1}, resulting in a median lethal toxicity (LC50) for *C. elegans* of 6.7 μg mL^{-1} (6 day exposure at 25 °C; personal communication Raffi Aroian). Before use, the protein was pelleted at 14,000 rpm for 15 min and dissolved in M9 buffer (Na_2HPO_4: 6 g L^{-1}; KH_2PO_4: 3 g L^{-1}; NaCl: 5 g L^{-1}; $MgSO_4 \cdot 7H_20$: 3 g L^{-1}).

Dilution series for toxicity tests with Cry1.105, Cry2Ab2, Cry3Bb1, and Cry5B were prepared in 35 mM CAPS (cyclohexyl-3-aminopropanesulfonic acid), 50 mM CAPS and 15 mM bicarbonate buffer, and M9 buffer, respectively, yielding the following concentrations in the test: Cry1.105: 0.0015, 0.041, 0.081, 0.16, 0.33, 0.65, 1.3 μM; Cry2Ab2: 0.0016, 0.0098, 0.041, 0.082, 0.16, 0.33, 0.66, 1.3, 2.6 μM; Cry3Bb1: 0.0025, 0.010, 0.041, 0.081, 0.163, 0.33, 0.65, 1.3 μM; Cry5B: 0.0057, 0.011, 0.023, 0.046, 0.091, 0.18, 0.37 μM. As negative controls, 35 mM CAPS (Cry1.105 test), 50 mM CAPS (Cry2Ab2 test), 15 mM bicarbonate buffer (Cry3Bb1 test), and M9 medium (Cry5B test) without the respective Cry protein were used. Moreover, a 1 μM solution of Cry1.105 (0.5 μM in the test) was denatured by heat treatment (30 min at 80 °C) and used as additional negative control in a separate experiment.

Cry protein mixtures were prepared by merging equal volumes of equimolar solutions of Cry1.105, Cry2Ab2, and Cry3Bb1, yielding total protein test concentrations of 0.081, 0.16, 0.33, 0.65, and 1.3 μM. As the negative control, equal volumes of the three control buffer solutions used for the single exposure tests were mixed.

For toxicity comparisons between wild-type N2 and a mutant *bre-5* strain (see next section), Cry protein concentrations were chosen that were expected to inhibit reproduction of the wild type by 50–100%: 0.15, 0.49, 0.28 and 0.14 μM for Cry1.105, Cry2Ab2, Cry3Bb1, and Cry5B, respectively.

2.2. *Caenorhabditis elegans* strains

Caenorhabditis elegans var. Bristol, strains N2 (genotype: *C. elegans* wild type, DR subclone of CB original, Tc1 pattern I) and HY498 (genotype: *bre-5(ye17)* I) were obtained from the Caenorhabditis Genetic Center and maintained as stocks of dauer larvae (an alternative juvenile stage that occurs in response to a lack of food) on nematode growth medium (NGM) agar (per liter: 17 g bacto agar, 2.5 g bacto peptone, and 3 g NaCl; with the addition after autoclaving of 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 25 ml 1 M KH_2PO_4, and 1 ml of a solution containing 5 mg cholesterol mL^{-1}, prepared in ethanol; Brenner, 1974) following standard procedures (Sulston and Hodgkin, 1988; Lewis and Fleming, 1995).

The *bre-5* (bt-resistant) mutant strain is resistant to the nematicidal protein Cry5B (Marroquin et al., 2000). The *bre*-genes (*bre2* through *bre-5*) encode specific enzymes that synthesize glycosphingolipid (GSL) Cry protein receptors (Griffitts et al., 2005). The inability of the *bre-5* mutant to synthesize receptors for the Cry proteins leads to a lower susceptibility toward Cry5B.

2.3. Toxicity test with *Caenorhabditis elegans*

The nematode bioassay with *C. elegans* was carried out, with a few modifications, according to standard methods (ISO, 2010) as described in Höss et al. (2011). In the test wells (12-well polystyrene multidishes; Nunc, Wiesbaden, Germany), 0.5 ml of the Cry protein solution was mixed with 0.5 ml of a food medium which is *E. coli* suspension prepared in K-medium (3.1 g NaCl L^{-1}; 2.4 KCl g L^{-1}; Williams and Dusenbery, 1990) to achieve a bacterial density of 500 FAU (formazin absorption units).

Five first-stage juvenile worms (J1) were transferred to each test well and three replicates each were set up for the control and for the various concentrations. The initial length of the J1 worms was 312 μm (±43 μm, SD) in the test with Cry1.105 and Cry2Ab2 and 317 μm (±46 μm, SD) in the test with Cry3Bb1 and the mixed Cry proteins. After 96 h of incubation at 20 °C, the test was stopped by heat-killing the worms at approximately 50 °C. The samples were then mixed with 0.5 ml of an aqueous solution of rose Bengal (0.5 g L^{-1}), to stain the worms for easier counting, and stored at 4 °C until further use.

Nematode reproduction was quantified by counting the juvenile offspring under a dissecting microscope at 25 × magnification and dividing the total number of offspring by the number of introduced test organisms (offspring per test organism). Values for percent inhibition of reproduction compared to the respective negative control were plotted against the nominal protein concentrations. Data were fitted using a sigmoidal logistic model (Sigma Plot; Systat) to achieve concentrations–response curves, from which low and median effect concentrations (EC10 and EC50) were calculated. The lowest observed effect concentrations (LOEC) were determined by performing one-way analysis of variance (ANOVA) and Dunnett post-hoc tests using SPSS 15.0 (SPSS Inc.). No observed effect concentrations (NOEC) were defined as the next tested concentrations lower than the LOEC.

To test if the joint effect of the different Cry proteins in mixture can be regarded as concentration-additive, concentrations of the single proteins in the mixture were transformed to EC50-based toxic units (TU). The individual TUs for each protein were calculated according to Sprague (1970) by dividing its concentration in the

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