Article

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Subacute Effects of Maize-Expressed Vaccine Protein, *Escherichia coli* Heat-Labile Enterotoxin Subunit B (LTB), on the Springtail, *Folsomia candida*, and the Earthworm, *Eisenia fetida*

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The ecotoxicological effects of transgenic maize-expressed vaccine protein, *Escherichia coli* heat-labile enterotoxin subunit B (LTB), on two soil invertebrates were studied under laboratory settings. After being reared for 28 days on LTB-maize-treated soils, no apparent mortality of the springtail, *Folsomia candida*, or the earthworm, *Eisenia fetida*, was observed at levels well above conservatively projected estimated environmental concentrations. Therefore, it is concluded that there would be no acutely toxic effect of LTB to these species. As for the subacute effect, no significant differences of *F. candida* mean reproduction and *E. fetida* mean growth were observed between LTB-maize-treated samples and non-GM-maize-treated controls. In addition, no LTB was detected in the *E. fetida* whole-body extraction assay, which indicates there was no tendency for bioaccumulation. On the basis of these observations, it is predicted that any adverse effects of LTB-maize on *F. candida* and *E. fetida* would be minimal, if any.

KEYWORDS: *Escherichia coli*; enterotoxin subunit B; plant-made vaccine; transgenic maize; ecological effect; springtail; earthworm

INTRODUCTION

Recent developments in biotechnology make it possible to introduce genes across species and express various recombinant proteins in plant tissues. Among various plant-based biotechnologies, the production of pharmaceutical proteins using plants as a bioreactor represents a novel strategy of pharmaceutical production (*1, 2*). The potential advantages of such plant-made pharmaceutical (PMP) production, also called "biopharming" or "molecular farming", include low cost of large-scale production, no risk of animal pathogens, high storability and transportability, ability to express multiple proteins, and ability to be introduced into food crops for oral administration (*3*).

Escherichia coli heat-labile enterotoxin subunit B (LTB; 11.6 kDa) is a nontoxic subunit of diarrhea-inducing heat-labile enterotoxin (LT). Although LTB itself is not a hazardous substance, specific binding of homopentameric LTB to intestinal ganglioside GM_1 lets the enzymatically active A subunit of LT (LTA; 27.0 kDa) cross membranes, and then introduced LTA activates the G-protein-mediated signaling pathways, causing diarrhea symptoms (*4*). Because of its strong immunogenicity without adverse effects on human health, LTB has been widely studied as an oral vaccine against LT-induced diarrhea disease (*5*). In addition to its effectiveness as a vaccine, LTB is also known as a potent mucosal adjuvant, which enhances the mucosal immunogenicity of co-administered vaccines (*6*). For the establishment of safe, effective, and reasonable-cost application of LTB to human and veterinary health treatments, plant-based LTB production has been investigated in several plants, such as potatoes, maize, and tobacco (*7*). At Iowa State University, LTB production using a strain of transgenic maize that expresses LTB specifically in its kernel has been investigated (*8*). Oral administration of this LTB-expressing maize (LTB-maize) to mice induced strong mucosal and serum antibody responses (*9*−*11*). Therefore, future therapeutic application of this plant-made subunit vaccine is expected.

Although there are many potential advantages of PMPs that facilitate the development of this emerging technology, many uncertainties also exist about their effects on both human and environmental health. Therefore, clear risk assessments are required to recognize and mitigate PMP-induced hazards, so that issues of regulatory safety and public acceptance are effectively addressed (*12*−*15*). To quantitatively assess the risks associated with the unintended intake of maize-expressed LTB through food, Wolt et al. (*16*) conducted a comprehensive human health risk assessment and concluded there are minimal concerns because of no observed mammalian toxicity and limited exposure potential from confined production of LTB-maize.

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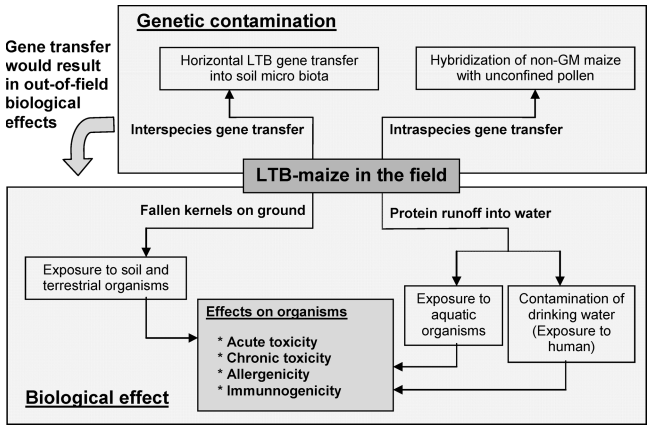

Figure 1. Conceptual model for the potential environmental concerns of LTB-maize.

Unlike human health risks, ecological impacts of transgenic maize-based LTB production are scarcely understood. As described in the conceptual model (**Figure 1**), potential ecological concerns over the open-field production of LTB-maize are diverse, ranging from genetic level contamination to more direct biological effects of recombinant proteins on nontarget organisms. Maize-expressed LTB produced in an open-field condition can potentially enter both the terrestrial and the aquatic environment through the spread of maize kernels by cultivation process and harsh weather, such as strong wind or flood, and its interactions with the surrounding ecosystem are of concern. In the case of Bt insecticidal protein expressed in transgenic maize tissues, its potential exposure to both terrestrial (*17*) and aquatic (*18*) ecosystems are studied. As for LTB, LTB-maize kernel left in the field is considered to be the most important route of environmental exposure, because it causes the greatest exposure of nontarget organisms to LTB at the highest concentration.

According to the U.S. EPA's *Guidelines for Ecological Risk Assessment* (*19*), the characterization of exposure and effects is required for comprehensive ecological risk assessment of stressors. Therefore, a quantitative understanding of the environmental fate and effects of maize-expressed LTB is necessary to determine any risks to the surrounding ecosystem. In this study, subacute effects of maize-expressed LTB on two important soil invertebrates, the springtail, *Folsomia candida*, and the earthworm, *Eisenia fetida*, were evaluated. Both *F. candida* and *E. fetida* are widely used for various soil ecotoxicological studies as good indicators of soil health, and there are many internationally accepted standard toxicological assay protocols using these species (*17, 20*). This study is expected to provide fundamental soil ecotoxicological profiles of maize-expressed LTB as an important step in the lower tier screening evaluation for the ecological risk assessment. Although many PMPs are highly bioactive and interaction with nontarget organisms ranging from soil microbes to larger mammals may occur, their ecological effects have not as yet been widely studied or reported. In anticipation of commercial-scale open-field PMP production, the development of standard methods to evaluate their ecotoxicological effects is needed. As a starting point for PMP ecotoxicological studies, the toxicological assay design developed in this study may be applicable to future ecological risk assessment for regulation of other PMPs.

MATERIALS AND METHODS

Estimation of Theoretical Field Exposure Concentration of Maize-Expressed LTB. Prior to the actual toxicological assays, theoretical field concentrations of maize-expressed LTB in soil were calculated using published data and conservative values. This estimated environmental concentration provides the appropriate rationale for dosing level in toxicological testing and the subsequent ecological risk assessment. In this study, the extreme worst-case scenario was used as the basis of estimated environmental concentration determinations.

Table 1. Proximate Analysis and LTB Expression Levels of Maize Kernels

	protein[a] (%)	fat[b] (%)	sugar[c] (%)	ash (%)	carbohydrate[d] (%)	moisture (%)	LTB (μg/g of kernel)
LTB-maize	11.7	6.28	1.43	1.52	71.3	9.21	30.17 ($\pm$3.01)
non-GM maize	9.64	5.04	1.38	1.27	73.8	10.2	0

[a] Protein, Dumas method. [b] Fat, acid hydrolysis method. [c] Sugar, HPLC. [d] Carbohydrate, subtract the added percentages of protein, moisture, fat, and ash from 100%.

Maize Materials. Transgenic maize *Zea mays* expressing *E. coli* heat-labile enterotoxin subunit B (LTB-maize) and a near-isoline, inbred B73, nontransgenic maize (non-GM maize) were used in this study. LTB-maize was developed and grown by the Iowa State University Plant Transformation Facility (Ames, IA) as described in Chikwamba et al. (*8*). This transgenic maize expresses immunogenically active pentameric LTB protein in kernels using a maize endoplasm-specific γ-zein promoter (*21*). Non-GM (B73) maize was obtained from the Iowa State University Raymond F. Baker Center for Plant Breeding (Ames, IA). As test materials, fourth-generation (R4) LTB-maize kernels cultivated in fall 2005 and non-GM maize kernels cultivated in fall 2006 were prepared. Naturally dried maize kernels were stored in the freezer at -20 °C until being used by February 2007. **Table 1** summarizes the nutritional profiles of both maize events. Nutritional analysis was conducted by Medallion Laboratories (Minneapolis, MN) using standard methods (**Table 1**) just before the start of the assays. For the entire study, finely ground dried maize kernels passed through 425 μm mesh openings were used. The use of ground maize kernels simplifies the experimental design and better assures the exposure of test organisms to LTB. Additionally, the use of ground kernels reduces the risk of any negligent spread of nonapproved germinatable transgenic seeds.

Measurement of LTB Expression Level in Maize. The LTB expression level in maize kernels was determined as described in Chikwamba et al. (*8*) with some modifications. Five hundred microliters of maize kernel extraction buffer [25 mM sodium phosphate buffer (pH 6.6), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% v/v Triton X-100, 10 μg/mL leupeptin, 0.25 mM Pefabloc SC] was added to 50 mg of finely ground maize kernels in 2-mL microcentrifuge tubes, and 10 replications were placed in a chamber at 37 °C for 2 h under gentle shaking. After 15 min of centrifugation at 13000*g*, supernatants were transferred to fresh 2-mL microcentrifuge tubes, and the amount of LTB in supernatants was quantified by ganglioside-dependent enzyme-linked immunosorbent assay (ELISA) as described below. For ELISA quantification, maize-extract samples were diluted with 1% dry milk in PBS [0.01 M Na_2HPO_4, 0.003 M KH_2PO_4, 0.1 M NaCl (pH 6.8$-$7.0)] to fall within the linear range of the standard curve.

ELISA. LTB in the extract was quantified by ganglioside-dependent ELISA as described previously (*9, 11*). A 96-well flat-bottom microplate (Thermo Fisher Scientific Inc., Waltham, MA) was coated with 2.5 μg/well of ganglioside GM_1 (Thermo Fisher Scientific Inc.) dissolved in sodium carbonate containing buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3 (pH 9.6)] for 1 h at room temperature. After the wells had been blocked for 1 h with 150 μL of 5% w/v dry milk in PBS, the LTB-containing samples were spiked directly to each well. At this step, specific binding of pentameric LTB to coated ganglioside GM_1 was occurring. After 1 h of incubation at 37 °C, LTB was detected by incubation with rabbit anti-LTB antibody (diluted 1:10000 in PBS with 1% w/v milk; Immunology Consultants Laboratory, Inc., Newberg, OR) for 1 h at 37 °C. To detect bound rabbit anti-LTB antibody, biotin-conjugated goat anti-rabbit IgG (diluted 1:5000 in PBS with 1% w/v milk; Sigma-Aldrich Co., St. Louis, MO) was spiked and incubated for 1 h at 37 °C. This second antibody was detected by incubation with streptavidin-conjugated horse radish peroxidase (diluted 1:1000; Thermo Fisher Scientific Inc.) for 30 min at room temperature, followed by an incubation with 3-ethylbenzthiazoline-6-sulfonic acid (ABTS;

Sigma-Aldrich Co.) substrate buffer [0.1 M citric acid, 0.55 M ABTS (pH 4.25)] for 30 min. Finally, the absorbance of reacted substrate was measured at 405 nm on a THERMOmax microplate reader with quantification by SOFTmax software (Molecular Devices Corp., Sunnyvale, CA). Sample volumes of 50 μL were used throughout the assay unless otherwise specified, and microplate wells were washed three times between each step using 300 μL of PBS with 5% v/v Tween-20 (PBST). Standard curves were prepared using bacterially produced LTB (purified by Dr. John Clements of Tulane University Medical Center, New Orleans, LA), and the linear range of the standard curve was 1.25−12 ng of LTB/mL.

Test Organisms. The springtail, *F. candida*, was obtained from the Oklahoma State University, Ecotoxicology and Water Quality Laboratory (Stillwater, OK). The test colony originated from Dr. Renate Snider of Michigan State University (East Lansing, MS), and the colony was maintained in our laboratory for 5 years prior to this assay. Culturing was performed on a mixture of hydrated $CaSO_4$ and activated charcoal in 950-mL glass Mason jars at 23 °C, and baker's yeast was provided as primal diet (*22*). *F. candida* is a small (<3 mm) parthenogenic arthropod living in humus-rich soil, and it can live up to 111−240 days depending on the soil temperature (*20*). To obtain a uniform growth stage of test organisms, approximately 30 springtails were placed in a newly prepared culturing bottle. After 4 days, adult springtails, which laid eggs on the fresh culturing medium, were removed from the bottle and the culturing bottle was maintained for 40 more days before collection of young springtails for the assay. Because eggs on the newly prepared media start hatching 7−10 days after being oviposited and offspring can start laying eggs 21−24 days after hatching (*20*), it is possible to collect only matured young springtails at 40 days after removal of the adult springtails. Thus, approximately 30-day-old mature springtails were used for the toxicological assay.

The earthworm, *E. fetida*, was purchased from a local vender and maintained for over 6 years in our laboratory. Culturing was conducted in 3-L rectangular containers filled with a 50:50 mixture of potting soil and horse manure. Horse manure used in this study was collected from a stable known to be free from medication, such as antibiotic treatments, and stored refrigerated at 3 °C until used. Earthworm-culturing containers were stored in a growth chamber under constant darkness at 23 °C, and the soil−manure mixtures were replaced every 3 months to remove wastes and supply new food. For earthworm assays, juvenile (<5 weeks after hatching) earthworms were used, because they may be more sensitive to toxicants compared to mature adult earthworms. To obtain a uniform earthworm growth stage, approximately 60 cocoons (egg cases) were collected from six different containers, and they were placed in a fresh culturing container with no earthworms. Five weeks after transfer of the cocoons, hatched juvenile earthworms were collected from the newly prepared container, and they were used for the assay. Because *E. fetida* has a long hatch time (*23*), similar size (7−20 mg) earthworms were randomly chosen to minimize the difference of initial age.

Soil Description. Agronomic surface soil from a reference maize field at the Iowa State University Agricultural Engineering/Agronomy Farm (Ames, IA) was used in this study. This field is confirmed to be free from pesticide application for more than 30 years. Field-collected soil was sieved moist through a 2-mm mesh steel sieve and stored at 4 °C until used. Test soil was classified as a mixture of the Webster and Nicollet soil with a sandy loam texture (47% sand, 36% silt, 17% clay) containing 2.6% organic matter. The soil pH was 6.8, and the moisture level was measured as 12.4%. Sieved soil properties were characterized by Midwest Laboratories Inc. (Omaha, NE) using standard methods. Prior to the assay, sieved soil was dried in an oven at 80 °C overnight to reduce soil fauna and was remoistened to 120% of field capacity with distilled water.

Springtail Survival and Reproduction Study. The springtail survival and reproduction study was conducted according to the method described in Clark et al. (*17*) for the ecotoxicological study of maize-expressed Bt Cry1 insecticidal proteins. The assay developed in Clark et al. (*17*) was primarily based on the ISO standard method (*24*) with modifications. In this assay, the 28-day survival and reproduction of springtails were chosen as endpoints to evaluate the subacute effect of maize-expressed LTB, and four different treatments (LTB-maize, non-GM maize, non-GM maize with pendimethalin, and no-maize) were prepared with 10 replications per treatment. For the assay, 25 g of soil was placed in loosely capped 4-cm (diameter) by 7-cm (height) glass jars, and 1 g of ground maize kernel (either LTB-maize or non-GM maize) was incorporated into each soil. This LTB-maize concentration was 5.2-fold higher than the maximum theoretical exposure level based on the worst-case scenario as described later. Because *F. candida* does not directly eat fresh maize kernels and feeds primarily on fungal hyphae grown in soil (*20*), maize−soil mixtures were aged for 14 days in a growth chamber under continuous darkness at 23 °C to facilitate decomposition of maize kernels before the start of the assay. As reference controls, 10 soil containers with non-GM maize were fortified to 2.25 mg of pendimethalin in 100 μL of acetone (90 μg of pendimethalin/g of soil), 1 day before the start of the assay. Pendimethalin is a dinitroaniline herbicide, and its toxicity to springtail has been characterized in previous studies (*17, 25*). In addition to three treatments with maize, a no-maize (soil only) treatment was also prepared. At the beginning of the assay, one springtail was randomly assigned to each jar, and all springtail-containing jars were kept in a room on a 16:8 light/dark cycle at 23 °C. Distilled water was sprayed every 3 days to keep the soil moisture near 20% w/w, by measuring the whole mass. From 3 to 5 grains of baker's yeast (<2 mg) were fed as the primary diet and replenished as consumed. After 28 days, all jars were flooded with a saturated sucrose solution to float springtails, which were then anesthetized by exposure to ethyl acetate fumes. Finally, the number of floated springtails was counted using a dissecting scope.

Earthworm Survival and Growth Study. The earthworm assay was conducted on the basis of the Clark et al. (*17*) and other standard methods (*26*−*29*). The 28-day survival and weight gain of juvenile earthworms were used as endpoints to observe the subacute effects of maize-expressed LTB. For the earthworm assay, a mixture of 15 g of soil and 10 g of horse manure was used as a culturing medium, and four treatments (LTB-maize, non-GM maize, non-GM maize with pendimethalin, and no-maize) with 10 replications were prepared. For maize assays, 500 mg of ground maize kernel (either LTB-maize or non-GM maize) was mixed into 25 g of soil−manure mixture in loosely capped glass jars (4-cm diameter by 7-cm height). This LTB-maize concentration was 2.6-fold higher than the maximum theoretical exposure level based on the worst-case scenario as described later. Because *E. fetida* relies more on the decomposed plant materials than fresh residues (*23*), sample containers were aged for 14 days in a growth chamber under continuous darkness at 23 °C to facilitate some decomposition before the start of the assay. A day before the addition of the earthworms, 22.5 mg of pendimethalin diluted in 200 μL of acetone was spiked to 10 non-GM-maize-containing soil−manure mixtures as reference controls (900 μg of pendimethalin/g of soil−manure mixture). The toxicity of pendimethalin to earthworm survival and growth was characterized in previous studies (*17, 25*). Additionally, 10 no-maize (soil−manure mixture only) treated samples were also prepared. Prior to the placement of one randomly selected juvenile (7−20 mg) earthworm into each jar, earthworms were cleaned in distilled water, blotted dry by paper towel, and weighed to record the initial mass. All earthworm-containing jars were placed in a growth chamber under continuous darkness at 23 °C, and the moisture level was maintained at approximately 30% w/w by measuring the whole mass every 7 days. After 28 days, earthworms were cleaned in distilled water and weights were recorded.

LTB Residue in Earthworm. Accumulation of maize-expressed LTB in earthworms was measured by homogenizing the whole body immediately after the 28-day weights had been recorded. Ten replications of LTB-maize-treated earthworms were homogenized individually with 1.4 mL of extraction buffer (50 mM sodium borate, 750 mM KCl, 10 mM ascorbic acid, 0.075% v/v Tween 20, pH treated to 9.5 by NaOH) in a glass tube using a Teflon homogenizer driven by a benchtop drill press. This extraction buffer was originally optimized for the extraction of LTB from soil (*30*). After 30 s of homogenization, the homogenizer head was rinsed with 0.4 mL of extraction buffer, and the homogenate was transferred to a 2-mL microcentrifuge tube. The sample-containing microcentrifuge tubes were shaken for 30 min at 200 strokes/min using a benchtop shaker (Shaker DO-10 L; ELMI Ltd., Riga, Latvia). After 5 min of centrifugation at 1500*g*, supernatants were transferred to fresh 2-mL microcentrifuge tubes, and LTB residues in the extracts were quantified by ELISA. In addition to earthworms treated

Table 2. Estimated Field Exposure Concentration of Maize-Expressed LTB in Soil

	abbrev	value	unit	ref
no. of ears per hectare	EpH	69000	ears/ha	*33*
no. of kernels per ear	KpE	400	kernels/ear	*31*
wt of kernel	WK	278	mg	*31*
LTB expression frequency		1	fraction	conservative value
kernel fallen frequency		1	fraction	conservative value
concn of LTB in kernel	CK	30.17	μg/g	measured value
bulk density of soil	BD	1.33	g/cm^3	*34*
depth of soil	DS	7.5	cm	conservative value
wt of soil per hectare	WSpH	997500	kg/ha	BD $\times$ DS $\times$ 10^5
wt of kernel per hectare	WKpH	7673	kg/ha	EpH $\times$ KpE $\times$ WK $\times$ 10^{-6}
wt of kernel per g of soil	WKpS	7.69	mg/g	WKpH/WSpH $\times$ 10^3
wt of LTB per g of soil	WLpS	232	ng/g	CK $\times$ WKpS

with LTB-maize, non-GM-maize-treated earthworms (negative control) and two different reference controls were each analyzed in three replications. As reference controls, either 100 or 500 ng of bacterially produced LTB diluted in 4 μL of purified water was injected into non-GM-maize-treated earthworms. After 30 min of aging at room temperature, earthworms injected with bacterial LTB were homogenized, and the recoveries of injected bacterial LTB were determined by ELISA. These reference controls show the reliability of the extraction method to determine the LTB residue in earthworms. A standard curve for ELISA quantification was prepared using a bacterially produced LTB diluted in extraction buffer.

Statistical Analysis. Statistical analysis was conducted by comparing the sample values to the negative control (non-GM-maize treatment) using Dunnett's ANOVA test. For the statistical comparison, individuals in each treatment that did not survive were excluded. All statistical analysis was conducted by JMP ver. 6.0 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

LTB Expression Level and Estimated Field Exposure Concentration. The expression level of LTB in transgenic maize kernels was measured as 30.17 μg of LTB/g of kernel, and no LTB was detected from non-GM-maize kernels (**Table 1**). On the basis of this LTB expression level, the theoretical field exposure concentration of maize-expressed LTB was estimated at 232 ng of LTB (7.69 mg of kernel)/g of soil as described in **Table 2**. This dose represents an extreme worst-case scenario with the following assumptions: (1) all maize kernels grown in the field are mixed into a 7.5 cm depth of soil; (2) maize-expressed LTB distributes uniformly into the soil; (3) LTB is expressed only in kernels; and (4) LTB is expressed uniformly in kernels at the level of 30.17 μg of LTB/g of kernel under 100% frequency of expression. Therefore, with fully conforming harvest practices described in Wolt et al. (*31*), the theoretical average environmental exposure of LTB-maize through harvest loss would be as much as 2684-fold lower than this value under worst-case scenario.

Springtail Survival and Reproduction Study. Over 80% of springtails survived in LTB-maize, non-GM-maize, and no-maize treatments (**Figure 2**). As presented in **Figure 3**, the 28-day average numbers of springtails in LTB-maize and non-GM-maize-treated samples were both 40 ($p = 1.0$, Dunnett's adjustment). In addition, no significant difference was observed between the non-GM-maize and the no-maize treatments ($p = 0.92$, Dunnett's adjustment). The fact that even newly hatched springtail offspring, which would be the most sensitive to stressors, could survive without any observed problems indicates that there were minimal effects of maize-expressed LTB on springtail survival and reproduction.

Pendimethalin treatment worked effectively as a reference control that suppresses the springtail survival and reproduction. The 28-day average survival of springtails was 30% in pendimethalin-treated soils, and no reproduction of surviving springtails was observed. This significant reduction in reference control proves the sensitivity of this assay to demonstrate the effect of LTB-maize on springtails.

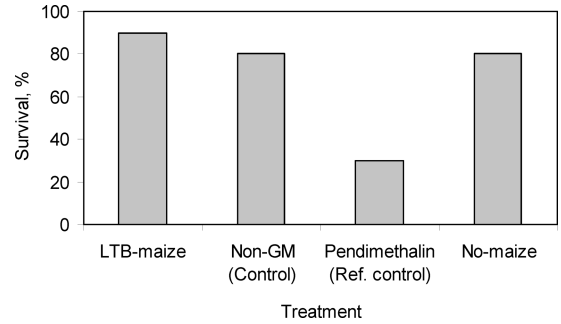

Figure 2. Survival of *F. candida* after 28 days of rearing on LTB-maize or three controls ($n = 10$).

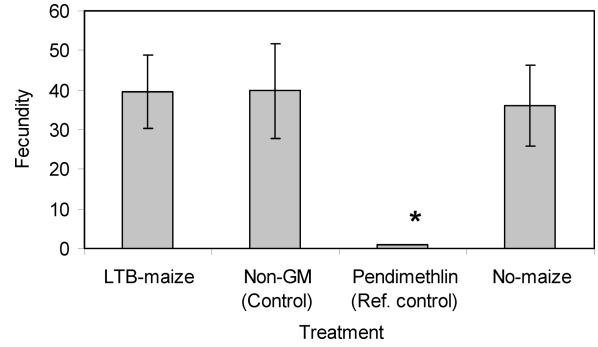

Figure 3. Average number of *F. candida* produced by surviving individuals after 28 days of rearing on LTB-maize or three controls. The asterisk indicates treatment statistically differs from non-GM-maize control ($p < 0.005$, Dunnett's adjustment).

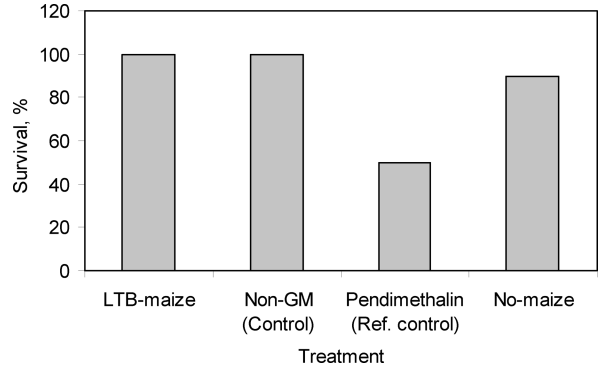

Figure 4. Survival of *E. fetida* after 28 days of rearing on LTB-maize or three controls ($n = 10$).

Because this is the first documented study to assess the effect of LTB on the soil ecosystem, no comparable data of LTB toxicity to the springtail are available. As a similarly designed transgenic maize ecotoxicological assay, Clark et al. (*17*) evaluated the effect of maize-expressed Bt Cry1Ab protein on the same colony of springtails using a similar experimental design. In their study, they concluded that there was no significant effect of maize-expressed Bt protein on springtail survival and reproduction, which was the same as observed in this study for LTB.

Earthworm Survival and Growth Study. After 28-days of incubation, no mortality of earthworms was observed in either the LTB-maize or the non-GM-maize-treated samples, and only one earthworm died in the no-maize treatment (**Figure 4**). These results indicate no acute toxicity of maize-expressed LTB on earthworms. The 28-day average change in biomass demon-

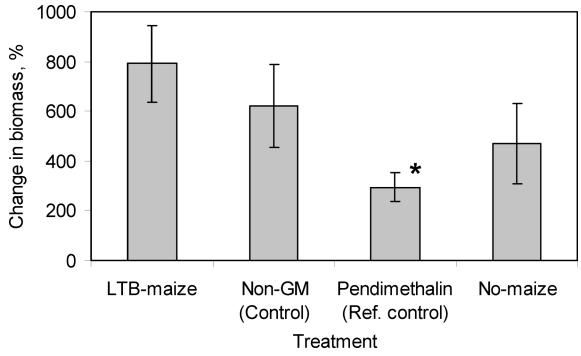

Figure 5. Average percentage change in mass for *E. fetida* surviving after 28 days of rearing on LTB-maize or three controls. The asterisk indicates treatment statistically differs from non-GM maize control ($p = 0.05$, Dunnett's adjustment).

strated no significant difference between the LTB-maize-treated and the non-GM-maize-treated earthworms (**Figure 5**; $p = 0.29$, Dunnett's adjustment). The average change in earthworm biomass between the non-GM maize treatment and the no-maize treatment also did not show any significant difference ($p = 0.52$, Dunnett's adjustment). However, the average change in biomass in the no-maize treatment was significantly lower than that in the LTB-maize treatment ($p = 0.02$, Dunnett's adjustment setting the no-maize treatment as the comparable control). Because no inhibition of the average growth of the sensitive juvenile stage earthworms was observed, it is concluded that there may be no adverse effect of maize-expressed LTB on the survival and growth of earthworms.

Survival of earthworms with pendimethalin treatment (reference control) was 50%, and the average change in biomass of surviving pendimethalin-treated earthworms was significantly lower than that of non-GM-maize-treated earthworms ($p = 0.05$, Dunnett's adjustment). This negative effect of pendimethalin addition showed the effectiveness of this assay to demonstrate the subacute effects of a stressor on earthworms.

As with the springtail assay, no previous data of LTB effects on earthworm survival and growth are available. In a related study on the transgenic maize ecotoxicity, Clark et al. (*17*) assessed the effects of maize-expressed Bt Cry1Ab protein using an experimental design similar to that described here. In their study, they concluded that there was no direct hazard from different strains of Bt-maize leaf material to earthworm survival and growth, the same as the result of this LTB-maize assay.

LTB Residue in Earthworm. No LTB residue was detected from the homogenates of earthworms cultured either in LTB-maize or in non-GM-maize-containing matrices. Because the digestive systems of earthworms would contain various digestive enzymes (*32*), it is expected that pentameric LTB ingested by earthworms would be cleaved or digested to undetectable forms instead of accumulating. This result also implies that maize-expressed LTB is not accumulatable through the food web. Reference controls injected with 100 and 500 ng of bacterial LTB showed 66.2 (±32.6) and 55.9 (±26.7)% recovery of injected LTB, respectively. This LTB spike recovery in reference controls indicates a capability to extract and quantify LTB in earthworms according to the method used in this study, although with low recovery percentage.

Ecological Risk Analysis Based on the Estimated Field Exposure Concentration. The ecological risk of maize-expressed LTB can be characterized as a function of exposure and effects (*19*). With regard to exposure, the environmental concentration of maize-expressed LTB was estimated to be 232 ng of LTB (7.69 mg of kernel)/g of soil. Because this estimation is based on the extreme worst-case scenario, this value can be recognized as the maximum field-exposure concentration. In comparison with the estimated environmental exposure concentration of 7.69 mg of kernel/g of soil, the amount of ground LTB-maize kernel used in the springtail assay (40 mg of kernel/g of soil) was 5.2-fold larger, and the amount used in the earthworm assay (20 mg of kernel/g of soil) was 2.6-fold larger. Because the doses used in assays were 2.6- or 5.2-fold larger than the maximum estimated concentration, it is considered that the initially applied LTB-maize concentration represents a conservative estimate of a field level of LTB.

For the purpose of exposure characterization, it is also important to know the persistence and fate of maize-expressed LTB. Proteins in soil are degraded by various factors, and this dissipation rate will be of consequence to the environmental dose to which nontarget organisms are exposed. In this study, test organisms were added to sample containers after 2 weeks of aging to facilitate decompositions of maize kernels and represent more realistic interaction with soil decomposers. Therefore, the actual amount of LTB existing in soil to which test organisms are exposed is predicted to be smaller than the initially applied value, due to protein degradation. In regard to the persistence of LTB expressed in the same stock of ground LTB-maize, we estimated that the DT_{50} (time for 50% dissipation) for extractable phase of maize-expressed LTB in the same soil used in this assay (the Webster-Nicollet soil) was 35−69 days depending on soil incubation temperature (*30*). Because different sample incubation conditions (i.e., amount of soil, moisture contents, addition of manure) were used to determine the DT_{50} for maize-expressed LTB, this DT_{50} cannot simply be applied to this ecotoxicological assay. However, considering this reference DT_{50}, it is predicted that a substantial amount of maize-expressed LTB still might have existed when test organisms were applied after the 2 week aging of maize samples.

Because no standard ecological risk assessment protocol for PMPs is established, the necessary duration of a chronic assay for each PMP needs to be judged on a case-by-case basis. In the case of LTB-maize, the 28-day assay is intended to provide a good comprehensive insight into possible ecotoxicological risks of LTB for the following reasons: (1) there was no apparent adverse effect observed in the 28-day assay; (2) maize confinement and harvest conditions will limit the environmental exposure potential; (3) the DT_{50} for maize-expressed LTB in soil was 1−2 months; and (4) LTB did not accumulate in the earthworm. These observations may minimize the need for the longer term chronic assay to assess the comprehensive risk of maize-expressed LTB on *F. candida* and *E. fetida*.

As a result of the risk analysis described so far, no evidence of LTB-maize-induced adverse effects on *F. candida* and *E. fetida* was observed. Therefore, direct toxicological effects of maize-expressed LTB on those species are expected to be negligible. Although no standard ecotoxicological assay protocol has been established for PMPs, the experimental method developed in this study is supposed to be applicable for the lower tier screening level assay of other PMPs in a soil ecosystem.

Future LTB Ecotoxicological Study Directions. Although the ecotoxicological effects of maize-expressed LTB were observed for two typical soil test organisms, its effects on numerous other organisms are unknown. For the ecological risk assessment, the selection of test organisms and assessment endpoints that adequately represent ecological risk is an important part of problem formulation (*19*). Although *F. candida* and *E. fetida* were selected as indicators of soil health for their wide adaptation to various soil ecotoxicological studies and ease of culturing, many other alternative test species could be considered. *F. candida* and *E. fetida* are

not maize-field oriented species, and maize is not a prime diet for them (*20, 23*). The use of maize-field oriented invertebrates that directly consume maize kernels may also be important, because they may be exposed to higher concentrations of maize-expressed LTB when they eat maize kernels. Because LTB-maize is strongly immunogenic to mammals, the potential toxicological and immunogenic effect of LTB-maize to wildlife, such as various rodents, should be also characterized. The soil ecosystem consists of numerous numbers of species from protozoa to larger mammals. Toxicity data on two invertebrates may be insufficient for the comprehensive ecological risk assessment, and more toxicological data on various species, from individual to population level, may be necessary before the large-scale production LTB and other PMPs can be begun.

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