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Full Length Research Paper

Quantification of Bt δ-endotoxins in leaf tissues of tropical Bt maize populations

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In Kenya, stem borers destroy an estimated 13.5% of farmers' annual maize harvest. Maize transformed using Bacillus thuringiensis (Bt) derived genes controls stem borers without negative effects to humans, livestock or the environment. The effectiveness and sustainability of Bt transgenic technology in the control of stem borers depends on the levels of concentration of the Bt δ-endotoxins in plant tissues. Kenya introduced Bt maize events to test the efficacy of Bt maize in controlling stem borers, and to develop high-yielding and locally adapted Bt maize germplasm for farmers. The objective of this study was to assess under greenhouse conditions the concentration levels of Bt δ-endotoxins in the leaf tissues of the parents, the F_1, and the $F_{2:3}$ populations of tropical maize, as a measure of stability and sustainability. Kenya introduced Bt maize events to test the efficacy of Bt maize in controlling stem borers, and to develop high-yielding and locally adapted Bt maize germplasm for farmers. The objective of this study was to assess under greenhouse conditions the concentration levels of Bt δ-endotoxins in the leaf tissues of the parents, the F1, and the F2:3 populations of tropical maize, as a measure of stability and sustainability. Two public Bt maize lines (Event 216 and Event 223) containing the *cry1Ab::ubi* gene were crossed with two non-Bt maize inbred lines, CML144 and CML159, to assess how the concentrations of Bt δ-endotoxins are transmitted from parents to F_1 and to F_2 generations. The mean concentration of Bt δ-endotoxins (µg/g) was 4.93 and 4.63 in Events 216 and 223 respectively. As expected, F_1 generations of all the crosses had similar concentrations of Bt δ-endotoxins. However, the F_2 generations showed a spread of concentrations. These findings may imply that genotypes with a higher mean concentration of Bt δ-endotoxins also have a lower level of plant damage traits expressed. In addition, these observations indicate that the *cry1Ab* gene was dominant and was inherited following the Mendelian segregation and that Events 216 and 223 could be utilized as reliable sources of resistance to stem borers in maize breeding programmes.

Key words: Bt maize, stem borers, Bt δ-endotoxins, enzyme-linked immunosorbent assay (ELISA), dot blot analysis, cry1Ab.

INTRODUCTION

Genetic engineering may create and preserve genetic diversity which can be exploited through conventional breeding (Ayad, 1997). The use of *Bacillus thuringiensis* (Bt) genes as sources of resistance is reported in literature for various crops and genotypes (Bravo et al., 2007). Maize breeding may require a systematic

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evaluation and introgression of genes for multiple borer resistance into locally adapted genotypes (Gethi et al., 2001; Bravo et al., 2007).

Nevertheless, the expression of transgenes depends on the genetic background (Bravo et al., 2007; Paris et al., 2008), and the physiological and environmental conditions (Lepri et al., 2002; Mahon et al., 2002; Olsen et al., 2005). The stability, inheritance and expression of transgenes in subsequent generations of breeding are of paramount importance for functional analysis as well as for crop improvement (Dietz-Pfeilstetter and Kirchner, 1998; Kathuria et al., 2003). Dutton et al. (2005) suggested that the stability of transgene expression is essential for transgenic crops to become an integral part of agricultural systems.

The effectiveness and sustainability of Bt transgenic technology in the control of stem borers will depend on the levels of expression of the Bt δ-endotoxins (Kranthi et al., 2005; Olsen et al., 2005; Dong and Li, 2007; Siebert et al., 2009). In addition, gene expression levels should be sufficient in appropriate plant tissues and express in successive generations (Kranthi et al., 2005; Olsen et al., 2005). In Kenya, stem borer species *Chilo partellus* and *Busseolla fusca* destroy an estimated 13.5%, of farmers' annual maize harvest. Maize transformed with a vector containing the Cry1Ab gene from a soil bacterium, Bt that produces insecticidal crystalline proteins, controls stem borers without any observable negative effects to humans, livestock or the environment. In 2010, biotech-nology crops occupied approximately 10% or 140 m ha^{-1} total of crop land. The aim of this study was to determine the levels of Bt δ-endotoxins retained in successive generations of crossing, breeding and seed production under biosafety greenhouse conditions.

MATERIALS AND METHODS

Research site and facilities

The study was conducted in 2007 and 2008 in the biosafety level II greenhouse complex (BGHC) and the biosafety laboratory located at the Kenya Agricultural Research Institute (KARI) Biotechnology Centre, at the National Agricultural Research Laboratories (NARL), Kabete. These facilities were designed and developed consistent with international standards and were approved for research on transgenic plants by the Kenya Plant Health Inspectorate Service (KEPHIS) on behalf of the Kenya National Biosafety Committee (NBC). The facilities are for bio-containment and provide an effective means of isolation and prevention of unintended transmission of genetic material (Traynor et al., 2001; Murenga et al., 2004; Mugo et al., 2005; Mugo et al., 2011a).

Maize parents, F_1 and F_2 generations

Plant materials for the study included the seedlings grown from the parents and the F_1 and F_2 generations of four populations (CML144 × Event 216, CML159 × Event 216, CML144 × Event 223, and CML159 × Event 223). Events 216 and 223 were obtained from CIMMYT - Mexico as BC_3S_1 lines. The two events were descended from a common parent, CML216, which was transformed with a vector containing a full-length cry1Ab coding sequence driven by an enhanced ubiquitin (cry1Ab::ubi) (Mugo et al., 2005). MBR C5 Bc F1-13-3-2-1-B-4-2-B is one of the many inbred lines developed from recombinations and recurrent selection usingmultiple borer resistance (MBR) populations under artificial infestation with various stem borer species (Mugo et al., 2001). Given that borers naturally occur in complexes, the MBR populations may be used in breeding programs to control more than one stem borer species. CKIR6009 is a maize hybrid formed from crosses of multiple borer resistant maize inbred lines, while H513 is a commercial maize hybrid in Kenya.

The genotypes were grown in pots filled with a planting medium composed of one part of topsoil mixed with farm yard manure, one part sand and one part coconut peat (Traynor et al., 2001; Murenga et al., 2004). The pots were irrigated twice a week to ensure vigorous growth. Other standard procedures for plant management at the BGHC level 2 were followed according to the laid down protocols (Murenga et al., 2004). F_1 generations were formed when twenty seeds each of Events 216 and 223, and CML144 and CML159 were sown in small transfer pots (7.5 × 7.5 × 9.0 cm) and later transplanted into large pots (12 × 30 cm). At anthesis, plants were cross-pollinated in predetermined combinations of Bt × Bt, Bt × non-Bt and non-Bt × non-Bt maize inbred lines. Bt plants and non-Bt plants were used as males and females, respectively. To ensure nicking, the sowing of the seeds was staggered over three different dates separated by 5 days. F_2 generations were formed from 20 F_1 plants through sib-mating with bulked pollen to an equal number of plants among other F_1 plants of the same cross. During anthesis, pollen from each cross were collected and bulked and used for pollinating an equal number of plants by sib-mating.

Experimental design and quantification of δ-endotoxins in leaf tissues

Seeds were sown and leaves from approximately 30 different plants were sampled 30 days after sowing for analysis of the levels of Bt δ-endotoxins. These plants were grouped into three categories of; resistant, moderate and susceptible plants based on the feeding damage found on the leaves. Three of the most recently fully-expanded leaves from each plant in the same generations were excised from the middle of the leaf blade, excluding the midrib, to standardize and minimize errors in leaf sampling (Dietz-Pfeilstetter and Kirchner, 1998). Protein was extracted from leaf tissue samples based on the procedure described by CIMMYT (2005). Dot blot analyses were used to confirm the presence or absence of biomolecules, which can be detected by DNA probes. For qualitative analysis of Bt in leaf tissues, 20 µl each of the extracted maize leaf samples was spotted and developed on a 0.2 µm nitrocellulose membrane as described by CIMMYT (2005).

The detection and quantification of Bt δ-endotoxins in maize leaf samples was carried out using the Bt cry1Ab/cry1Ac microtiter plate kit, a "sandwich-type" enzyme-linked immunosorbent assay (ELISA) (Greenplate, 1999). Leaf tissues from 20 plants from each parent and from all their crosses in the different generations were sampled and ground in liquid nitrogen. This was followed by homogenization in 5 ml of 0.1 M sodium bicarbonate pH 10.01 containing 10 mM 2-mercaptoethanol, 2.5 mM EDTA, 2.5 mM EGTA, 1 mM benzamidine-HCl, 0.5 mM PMSF, 1 µg/ml pepstatin A, 40 µg/ml bestatin, 1 mM CWS, and 10% (v/v) glycerol. In the assay system, the Cry1Ab standards, controls, or sample extracts loaded into wells coated with monoclonal antibodies raised against

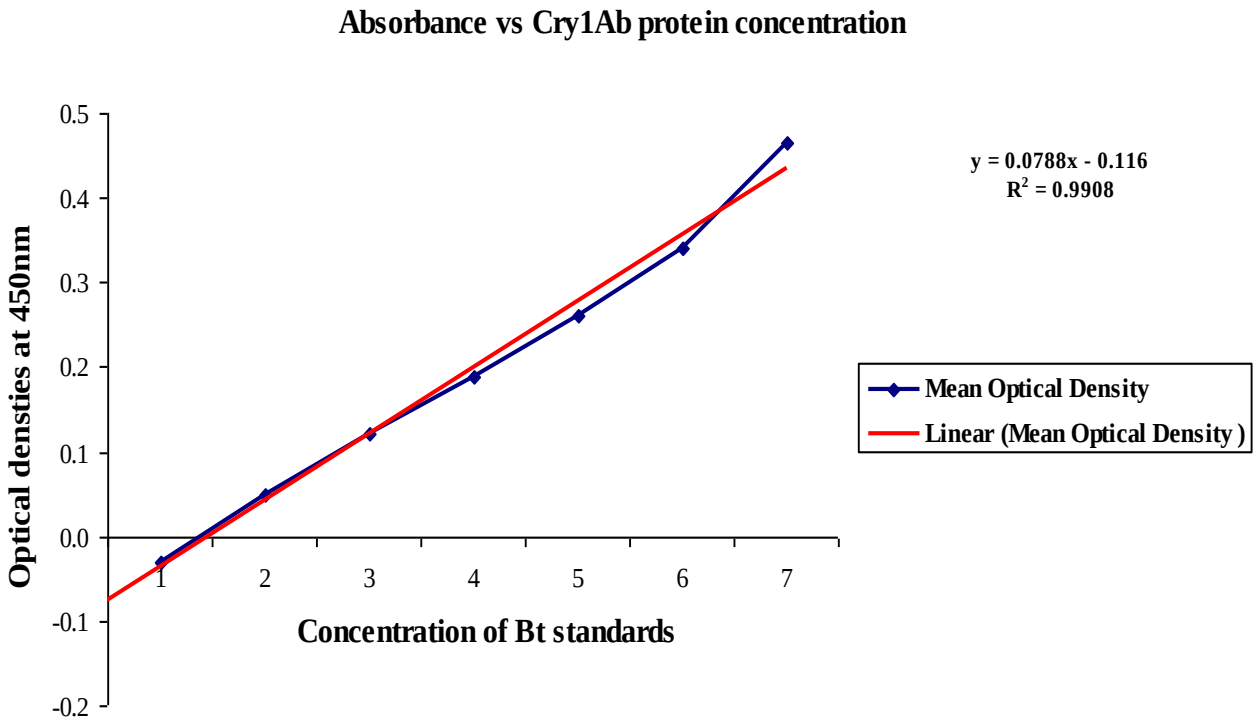

Figure 1. Absorbance versus Cry1Ab protein concentration (µg/g) from the standards.

Cry1Ab proteins. Any residues of Bt δ-endotoxins found in the standard or sample extracts is bounded to the antibodies on the wells. The "sandwich" ELISA was completed by the addition of immunoaffinity-purified polyclonal goat-antibodies specific to the same Bt δ-endotoxins. A dose response curve of absorbance of the colored product formed versus concentration was generated using results obtained from Cry1Ab protein standards from the ELISA kit (Figure 1).

Data analysis

ELISA data from parents, F_1 and F_2 generations were subjected to analysis of variance (ANOVA), and the means were computed and separated using t-tests (LSD), for each experimental data set at (P = 0.05). Contrast analyses of the same data for the adjusted mean concentration of Bt δ-endotoxins were carried out on the parents, their crosses and successive generations.

RESULTS

Quantification of Cry1Ab protein levels using the ELISA test

Analysis of leaf tissues using ELISA indicated significant differences among generations with respect to Cry1Ab protein content (Table 1). The Bt parents (Events 216 and 223) had mean concentrations of Bt δ-endotoxins of 4.93 and 4.63 µg/g, respectively. Observations of 30 F_1s of Event 223 × Event 216 showed no significant differences ($p<0.496$), with a mean concentration of 4.35 µg/g. The 30 F_2s of CML144 × Event 216 showed highly significant differences ($p<0.0001$), with a mean concentration of 1.99 µg/g. No significant differences ($p<0.3830$) in the concentration of Bt δ-endotoxins were found among 30 CML144 × Event 216 F_1s. These crosses had a mean of 4.24 µg/g. Nevertheless, highly significant differences ($p<0.0001$) were observed among crosses of 30 CML144 × Event 216 F_2s, with a mean concentration of 3.90 µg/g. Similarly, no significant differences ($p<0.3833$) were observed for 30 CML144 × Event 223 F_1s, with a mean concentration of 3.92 µg/g. However, highly significant differences ($p<0.0001$) among 30 CML144 x Event 223 F_2's were observed, with a mean concentration of 1.69µg/g. No significant differences in the concen-tration of Bt δ-endotoxins were observed among 30 CML159 x Event 216 F_1s ($p<0.7058$), with a mean of 5.44 µg/g.

However, highly significant differences ($p<0.0001$) were reported among the 30 CML159 × Event 216 F_2s, with a mean concentration of 1.60 µg/g (Table 1). Thirty CML159 × Event 223 F_1s were not significantly different ($p<0.7058$), with a mean concentration of 5.44 µg/g (Table 1). Nevertheless, highly significant differences ($p<0.0001$) were observed among 30 CML159 x Event 223 F_2's with a mean concentration of 1.60µg/g (Table 1).

Table 1. Mean concentration of Bt δ-endotoxins (µg/g) of leaf tissue recorded from parents, F_1 and F2 populations of crosses CML144 × Event 216 and CML159 × Event 216.

Genotype	Mean concentration of Bt δ-endotoxins (µg/g) of leaf tissue
Parents	
CML144	0.00[c]
CML159	0.00[b]
Event 216	4.93[a]
Event 223	4.63[a]
Checks	
CKIR6009	0.00[a]
H513	0.01[a]
MBR	0.02[a]
CML216	0.00[a]
F_1 populations	
Event 223 × Event 216	4.35[e]
CML144 × Event 216	4.24d[e]
CML144 × Event 223	3.92d[e]
CML144 × CML159	0.00[a]
F_2 populations	
Event 223 × Event 216	1.99cd[e]
CML144 × Event 216	3.90cd[e]
CML159 × Event 223	3.38d[e]
CML144 ×CML159	0.00[a]

Means are tested with t tests (LSD) for Bt x Bt, Bt x non-Bt and non-Bt x non-Bt crosses at the p=0.05. Values followed by the same letter are not significantly different in the same column.

Thirty of both F_1s and F_2s of CML144 × CML159 had no significant differences (p<0.9675), with a mean concentration of 0.00 µg/g. Contrasts of adjusted mean concentrations of Bt δ-endotoxins in leaf tissues indicated highly significant differences (P<0.0001) among Bt maize Events 216 and 223 and CML144 and 159, and their F_1 and F_2 generations of crosses of tropical maize (Table 2). The results of the contrast analysis show highly significant differences between the Bt parents and the non-resistant parents (p<0.0001). A contrast of Events 216 and 223 versus all F_1 generations showed no significant differences (p>0.4960). They have highly significant differences (p<0.0001) between the F_1 generations compared to all the crosses of Bt maize events in the F_2 generations.

DISCUSSION

The analysis of all F_1s and F_2s of the Bt × Bt crosses showed significant differences in the mean concentration of Bt δ-endotoxins. Neither were there any significant differences among the Bt × non-Bt crosses for all F_1s. However, the F2s showed significant differences in all the crosses, probably due to Mendelian segregation. These results agree with the findings by Kranthi et al.(2005) in Bt cotton, and Sulistyowati et al. (2008) in their studies on segregation patterns of insect resistance genes in the progenies and crosses of transgenic rojolele rice. The study findings are consistent with the Mendelian inheritance of a single dominant locus such as the *Cry1Ab* gene. In addition, these results suggest that in the leaf tissues, expression of the concentration of Bt δ-endotoxins was inherited stably and follows the Mendelian segregation as observed in the crosses of Bt maize events and the non-Bt F_2 generations (Siebert et al., 2009; Mugo et al., 2011).

In the contrast analysis, crosses between Bt and non-Bt F_1 generations had no significant effect on the mean concentration of Bt δ-endotoxins. This may imply that the

Table 2. Contrasts of adjusted mean concentrations of Bt δ-endotoxins among various segregating generations of crosses of tropical maize.

Source	DF	Mean square
Treatment	19	37.762***
Plant*treatment	31	2.690^{ns}
Contrasts		
Event 216 vs. CML144 and CML 159	1	84.369***
Event 223 vs.CML144 and CML 159	1	47.778***
Events 216 and 223 vs. CML144 and CML 159	1	85.977***
Events 216 and 223 vs. (Event 216 x 223) F_1	1	0.889^{ns}
Events 216 and 223 vs. (CML144 x CML159) F_1	1	161.952***
Event 216 vs. All F_1s	1	2.239^{ns}
Event 223 vs. All F_1s	1	0.273^{ns}
Events 216 and 223 vs. All F_1s	1	1.459^{ns}
Event 216 vs. All F2s	1	36.724***
Event 223 vs. All F_2s	1	13.010**
Events 216 and 223 vs. All F_2s	1	38.035***
(Event 216 × 223) F_1 vs. All F_1s	1	0.989^{ns}
(CML144 × CML159) F_1 vs. All F_1s	1	265.103***
(Event 216 × 223) F_1 vs. All F_2s	1	24.973***
(CML144 × CML159) F_1 vs. All F_2s	1	126.831***
(Event 216 × 223) F_2s vs. All F_2s	1	9.232^{ns}
(CML144 × CML159) F_2s vs. All F_2s	1	48.846***

***=Very highly significant, **= highly significant, *= significant.

gene was stably integrated into the genome of the F_1 crosses (Zhu et al., 2004). It may also suggest that the transgene could be successfully transferred from Bt maize lines to non-Bt maize lines and that the Cry1Ab gene was dominant. The highly significant differences observed among the crosses of Bt and non-Bt F_2 generations may be attributed to Mendelian segregation.

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REFERENCES

Bravo A, Gill SS, Soberón M (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon. 49: 423-435.

CIMMYT (2005). Laboratory Protocols: CIMMYT Applied Molecular Genetics Laboratory. 3rd ed., Mexico, DF: CIMMYT.

Dietz-Pfeilstetter A, Kirchner M (1998). Analysis of gene inheritance and expression in hybrids between transgenic sugar beet and wild beets. Mol. Ecol. 7: 1693-1700.

Dong HZ, Li WJ (2007). Variability of Endotoxin Expression in Bt Transgenic Cotton. J. Agron. Crop. Sci. 193: 21-29. DOI: 10.1111/j.1439-037X.2006.00240.x.

Dutton A, Romeis J, Bigler F (2005). Effects of transformed maize lines carrying Bt genes expresing *cry1Ab* and Bt spray on *Spodoptera littoralis*. Entomologia experimentalis et applicata, 114: 161-169.

Gethi M, Mutinda C, Diallo A (2001). Stem borers in maize: A natural stress and progress towards host plant resistance. Seventh Eastern and Southern Africa regional maize conference. pp. 45-48.

Greenplate JT (1999). Quantification of Bacillus thuringiensis insect control protein Cry1Ac over time in Bollgard® cotton fruit and terminals. J. Econ. Entomol. 92: 1377-1383.

Kathuria H, Mohanty A, Tyagi KA (2003). Analysis of inheritability and expression profile of single and multi-copy transgenes in rice over generations. J. Plant Biochem. Biotechnol. 12: 103-107.

Kranthi KR, Naidu S, Dhawad CS, Tatwawadi A, Mate K, Patil E, Bharose AA, Behere GT, Wadaskar RM, Kranthi S (2005). Temporal and intra-plant variability of *cry1Ac* expression in Bt cotton and its influence on the survival of the cotton bollworm *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera). Curr. Sci. 89(2): p. 291.

Lepri B, Thu H, Christou C (2002). Endogenous enzyme activities and polyamine levels in diverse rice cultivars depend on the genetic background and are not affected by the presence of the hygromycin phosphotransferase selectable marker. Theor. Appl. Genet. 105: 594-603. DOI: 10.1007/s00122-002-0922-4.

Mahon R, Finnegan J, Olsen K, Lawrence L (2002). Environmental stress and the efficacy of Bt cotton. Australian Cottongrower, 22: 18-21.

Mugo S, De Groote H, Bergvinson D, Mulaa M, Songa JMSG (2005). Developing Bt maize for resource poor farmers - recent advances in the IRMA project. Afr. J. Biotechnol. 4: 1490-1504.

Mugo S, Gichuki S, Murenga M, Taracha C, Macharia H (2011a). Experiences with the biosafety regulatory system in Kenya during the introduction, testing and development of Bt maize. Afr. J. Biotechnol. 10: 4682-4693.

Mugo SM, Murenga MG, Taracha C, Songa JM, Gichuki S, Tende R, Karaya H, Bergvinson DJ, Pellegrinesch A, Hoisington DA (2011). Testing public Bt maize events for control of stem borers in the first confined field trials in Kenya. Afr. J. Biotechnol. 10:4713-4718.

Murenga GM, Mugo SM, Odhiambo BA, McLean SCT (2004). Manual for biosafety level 2 greenhouse for research on transgenic plants at KARI Biotechnology Centre, Insect Resistant Maize for Africa (IRMA) Project document No. 14, KARI and CIMMYT, Nairobi, Kenya,

Olsen KM, Daly JC, Finnegan EJ, Mahon RJ (2005). Changes in *cry1Ac* Bt transgenic cotton in response to two environemntal factors. J. Econ. Entomol. 98: 1382-1390.

Paris M, Roux F, Berard A, Reboud X (2008). The effects of the genetic background on herbicide resistance fitness cost and its associated dominance in *Arabidopsis thaliana*. Heredity, 101: 499-506.

Siebert MW, Patterson TG, Gilles GJ, Nolting SP, Braxton LB, Leonard BR, Van Duyn JW, Lassiter RB (2009). Quantification of Cry1Ac and Cry1F Bacillus thuringiensis Insecticidal Proteins in Selected Transgenic Cotton Plant Tissue Types. J. Econ. Entomol. 102: 1301-1308.

Sulistyowati YS, Hartana A, Inez HS (2008). The segregation pattern of insect resistance genes in the progenies and crosses of transgenic rojolele rice. Indonesian J. Agric. Sci., 9 (2): 35-43.

Traynor PL, Adair D, Irwin R (2001). A practical guide to containment. Greenhouse research with transgenic plants and microbes, In: Traynor PL (Eds.). Information System for Biotechnology, Virginia Tech, Blacksburg, VA., at http://www.isb.vt.edu/cfdocs/greenhouse_manual.cfm, Information System for Biotechnology, Virginia Tech.

Zhu B, Lawrence JR, Warwick SI, Mason P, Braun L, Halfhill MD, Stewart CN (2004). Stable Bacillus thuringiensis (Bt) toxin content in interspecific F1 and backcross populations of wild Brassica rapa after Bt gene transfer. Mol. Ecol. 13: 237-241.