R E P O R T S

Fig. 5. Southern blot of susceptible (S) and resistant (R) strain individuals showing multicopy occurrence of Hel-1. Genomic DNA was digested with Apa I, electrophoresed in a 0.8% agarose gel, blotted to nylon membranes, and probed with radiolabeled Hel-1 LTR.

involved in cell adhesion (14). Whatever its function, it is not essential for life, because YHD2 is viable and fertile under laboratory conditions despite being a “natural knockout” for HevCaLP. Whether its absence confers a fitness disadvantage in the field has important implications for resistance management, and this question can now be addressed with the information developed here.

These results suggest a new interpretation of our previous estimate of 0.0015 for the frequency of YHD2-type resistance alleles in field populations of H. virescens before widespread planting of Bt cotton (15). In that study, field-caught males were individually mated to homozygous resistant YHD2 virgin females, and their progeny were tested at a discriminating dose of Cry1Ac-containing diet. The majority of males were homozygous susceptible, as expected, producing only heterozygous progeny that did not grow on Cry1Ac because the resistance-conferring effect of r1 is recessive. However, 3 of 1025 males were heterozygous, producing some progeny that did grow on the Cry1Ac diet because they inherited the r1 allele from their YHD2 mother and a field-derived resistance allele from their father.

Our previous interpretation implicitly assumed that the paternally contributed resistance allele was also r1. But it is now evident that any other allele with a molecular lesion somewhere in HevCaLP preventing it from functioning as a lethal target would give the same result, because r1 is a null allele. Thus, 0.0015 actually represents a frequency estimate of the entire class of such defective HevCaLP alleles. This statement applies even if r1 itself does not occur in the field but arose in the lab. Thus, the development of efficient DNA-based methods to detect other types of mutants at BtR-4 should be a high priority. Screening solely for the Hel-1 insert detects r1 but may underrepresent the total frequency of resistance alleles in the field.

Monitoring resistance allele frequencies in field populations will enable a direct test of whether the high-dose/refuge strategy is succeeding. If it starts to fail, detection of increasing heterozygote frequencies will indicate that a problem is looming, well before resistant homozygotes become frequent enough to cause uncontrollable outbreaks. This may allow enough time for the strategy to be adjusted to reverse the increase. We thus suggest that allele frequency monitoring be incorporated into resistance risk assessment. At the very least, preservation of DNA samples should accompany existing bioassay-based monitoring programs. Even if other Bt resistance genes are later discovered in H. virescens, any delay in initiating BtR-4 allele monitoring erodes the opportunity to make informed modifications to a strategy that could sustain the use of Bt transgenics and prolong their environmental benefits of reducing dependency on conventional insecticides.

References and Notes
18. GenBank accession numbers AF367362 and AF367363. Prediction methods and alignment with BTR1 and BTR175 are documented in supplementary material available at Science Online (www.sciencemag.org/cgi/content/full/293/5531/857/DC1).
19. We thank A. Westman, D. Bourguet, and C. Robin for helpful comments on the manuscript. Supported by NSF grant MCB-9816056.
22 March 2001; accepted 15 June 2001

Bt Toxin Resistance from Loss of a Putative Carbohydrate-Modifying Enzyme
Joel S. Griffiths, Johanna L. Whitacre, Daniel E. Stevens, Raffi V. Aroian*

The development of resistance is the main threat to the long-term use of toxins from Bacillus thuringiensis (Bt) in transgenic plants. Here we report the cloning of a Bt toxin resistance gene, Caenorhabditis elegans bre-5, which encodes a putative β-1,3-galactosyltransferase. Lack of bre-5 in the intestine led to resistance to the Bt toxin Cry5B. Wild-type but not bre-5 mutant animals were found to uptake toxin into their gut cells, consistent with bre-5 mutants lacking toxin-binding sites on their apical gut. bre-5 mutants displayed resistance to Cry14A, a Bt toxin lethal to both nematodes and insects; this indicates that resistance by loss of carbohydrate modification is relevant to multiple Bt toxins. Crystal toxins produced by B. thuringiensis are used worldwide in transgenic crops to control caterpillars and beetles, are an important tool of organic farming, and have made important contributions to the control of insect-borne diseases such as African river blindness. Once ingested by an insect, Bt toxins are proteolytically activated in the midgut and bind to membrane gut receptors, leading to pore formation and death (1, 2). Although Bt toxins are safe to vertebrates and are considered beneficial to the environment relative to chemical pesticides, Bt toxin effectiveness is threatened in the long term by the development of insect resistance (3). Bt-resistant variants of the diamondback moth have been identified in the field, and resistant strains of at least 11 insect species have been documented in the laboratory (4, 5). Understanding the molecular mechanism of toxin action and identifying the genes that can mu-
tate to yield resistance are important steps in developing strategies to help delay or circumvent this problem.

Some Bt toxins are toxic to the nematode C. elegans (6). The best characterized of these nematicidal toxins, Cry5B, falls into a phylogenetic group of eight Bt toxins, at least two of which, Cry5A and Cry14A, are toxic to insects (2). Cry5B has ~24% sequence identity to commercially important insecticidal toxins, such as Cry1Ac, in the toxin domain. Cry5B contains four of the five protein sequence blocks conserved among most Bt toxins and may fold into a three-domain structure related to those of insecticidal toxins (1, 7). Like insects, nematodes fed Bt toxin rapidly cease feeding and incur intestinal damage (6, 8). These similarities in toxin sequences and response suggest that the general mechanism of Bt toxicity in nematodes and insects is conserved. By studying the process in C. elegans, we can take advantage of the molecular, genetic, and cell biological tools available in this model organism. In addition, nematicidal Bt toxins are important to study because of their potential to control plant-parasitic nematodes. These widespread

Fig. 1. BRE-5 encodes a putative galactosyltransferase that is required in the C. elegans gut for Bt toxin action. (A and B) Rescue experiments. In (A), a bre-5(ye17) animal fed Bt toxin for 24 hours shows a healthy, resistant gut. In (B), a bre-5(ye17) animal transformed with the 4.3-kb rescuing fragment fed Bt toxin for 24 hours shows a damaged, susceptible gut. Anterior is to the left. The posterior pharynx and anterior intestine are shown for each animal. Scale bar, 50 μm. (C) CLUSTALW (version 1.81) alignment of BRE-5 protein with human β-1,3-galactosyltransferase polypeptide 5 (hB3T5), mouse β-1,3-galactosyltransferase polypeptide 3 (mB3T3), and Drosophila BRAINIAC (Brn). Blue, absolutely conserved residues; green, conserved amino acid groups. The putative transmembrane domain is underlined; the DxD and DDVFGT motifs are double-underlined (single-letter abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr). The locations of the two arginines mutated in the bre-5 alleles are indicated: ye107 alters an arginine conserved in all β-1,3-galactosyltransferases; ye17 introduces a premature stop codon. (D and E) Mosaic analysis showing that lack of BRE-5 in the gut leads to resistance to Bt toxin. Orientations are as in (A) and (B); scale bar, 50 μm. Arrows point to the anterior end of the gut. Exposures were identical for each pair of wild-type and bre-5(ye17) images. Shown in (D) are differential interference contrast (DIC) and deconvolved fluorescein isothiocyanate (FITC)-channel images of a bre-5(ye17) animal transformed with the extrachromosomal array that contains the 4.3-kb rescuing fragment (the animal is therefore sensitive to toxin) and SUR-5GFP(NLS), which results in GFP expression in the nuclei. Five large gut nuclei are in focus and brightly express GFP (arrowheads). In (E), DIC and deconvolved FITC-channel images of a mosaic animal that expressed GFP in some cells (and therefore contained the array) but was resistant to toxin (and therefore had lost the array in cells required for toxicity) are shown. In all 67 resistant animals, the gut nuclei never express GFP, indicating that the array was lost in the gut lineage. In the animal shown, the array is present in pharyngeal terminal bulb cells, descendants of the MS lineage.
agricultural pests cause $\sim$80 billion per year in crop damage (9) that is likely to be exacerbated by an upcoming worldwide ban of methyl bromide, the main chemical currently used to control them.

We previously reported the identification of five C. elegans genes, called bre (for Bt resistance), that mutate to Cry5B resistance (6). One of these genes, bre-5, was mapped to the right end of the chromosome IV cluster. We transformed bre-5 mutant animals with cosmids in this area and rescued toxin resistance to toxin susceptibility in these animals with the cosmids T12G3 (10). We then narrowed down bre-5 rescue to a 4.3-kb fragment within T12G3 (Fig. 1, A and B). This fragment did not contain any genes predicted in the genomic database, but it did contain a single potential gene with extensive sequence similarity to mammalian glycosyltransferases. The cDNAs corresponding to this gene were isolated and a complete sequence assembled (11); it encodes a 322–amino acid protein (Fig. 1C). To confirm that this gene is bre-5, we sequenced the complete bre-5 coding region from each of our two bre-5 mutant alleles. Both alleles show alterations in this coding region consistent with a loss or reduction of function (Fig. 1C).

BLAST and protein domain searches indicated that BRE-5 is a member of the β-1,3-galactosyltransferase family that transfers galactose onto proteins and lipids (12). BRE-5 is most similar in sequence to the Drosophila protein BRAINIAC. Over a 200–amino acid stretch that includes the catalytic domain, BRE-5 shows 37%, 27%, and 25% sequence identity to BRAINIAC, mouse β-1,3-galactosyltransferase 3, and human β-1,3-galactosyltransferase 5, respectively (Fig. 1C). BRAINIAC has been implicated in Notch signaling, perhaps by influencing ligand-receptor interactions, as has been speculated (13, 14). BRE-5 contains all the hallmarks of β-1,3-galactosyltransferases, including a putative NH2-terminal transmembrane domain, an Asp-X-Asp motif, and a conserved variant of the Glu-Asp-Val-Tyr-Val-Gly motif. To confirm that loss of this galactosyltransferase gene leads to Bt toxin resistance, we injected hermaphrodite gonads with double-stranded (ds) RNA derived from the bre-5 cDNA (15). Injection of dsRNA is known to deplete gene function in the progeny of injected hermaphrodites via RNA interference (RNAi) (16). After injections of dsRNA at 1.5 and 3.0 mg/ml, we found that, respectively, 45% (n = 60) and 73% (n = 60) of the progeny were resistant to Cry5B. These results confirm that Bt toxin resistance is the bre-5 loss-of-function phenotype. As previously reported for bre-5(yle17) (6), we did not detect lethality or other obvious phenotypes in bre-5 RNAi animals.

On the basis of the identification of BRE-5 as a putative galactosyltransferase and numerous in vitro studies that pointed to the importance of carbohydrates in the binding of insecticidal Cry1Ac to receptor and membrane (17, 18), we hypothesized that BRE-5 functions in forming a carbohydrate structure, present on proteins or lipids exposed at the gut surface, that is necessary for toxin binding. In the absence of bre-5–dependent carbohydrates, Bt toxin cannot bind, resulting in resistance. Such a requirement for carbohydrates in microbial toxin recognition would not be without precedent. For example, cholera toxin binds to host cells via carbohydrates (19).

To understand BRE-5 function better, we performed experiments involving mosaic animals to determine the anatomical focus of the gene’s function with respect to Bt toxin susceptibility. Homozygous bre-5(yle17) hermaphrodites were injected with a cocktail of plasmids that included the dominant rol-6 marker (which causes animals to roll), SUR-5GFP(NLS) (which is expressed in the nuclei of many somatic cells, including the intestine (20), and the 4.3-kb rescuing fragment of bre-5. A stable line was established that transmitted all three transgenes as an extrachromosomal array in 50% of the progeny. As a result of bre-5 rescue and expression of SUR-5GFP, rolling worms were sensitive to Cry5B, as expected, and displayed nuclear green fluorescent protein (GFP) (Fig. 1D). Of 2060 worms that were rolling (and therefore carrying the array) and were transferred to toxin plates, 67 rare, toxin-resistant animals were identified. These animals were resistant presumably because the extrachromosomal array had been lost during somatic divisions (20) in the tissue(s) where BRE-5 expression is needed for toxin to be effective. When these resistant, mosaic animals were examined for fluorescence, all 67 had lost GFP signal in the gut (Fig. 1E), indicating that the array and BRE-5 function were missing in the gut lineage, derived exclusively from the E blastomere. Furthermore, in 19 of these 67 animals, GFP staining still was present in posterior pharyngeal cells derived from MS, the sister of E (Fig. 1E), excluding the possibility that the array also had to be lost in other cells leading up to the birth of the E cell. We have verified that nonrolling mosaic animals also lack GFP fluorescence in the gut (20 of 20 animals). Thus, loss of the wild-type bre-5 gene in the gut causes animals to be resistant to toxin. Consistent with this finding, we have performed immunofluorescence with a recently purified BRE-5 antibody that indicates expression in the gut (21).

To test directly whether BRE-5 is required for toxin to interact with the nematode gut in vivo, we fed fluorescently labeled Bt toxin to L4-staged hermaphrodites and followed its fate in wild-type and bre-5 mutant animals (22). In wild-type animals, labeled toxin was internalized by gut cells, where it colocalized with autofluorescent gut granules, probably the site of the intestinal lysosome (23) (Fig. 2, A and B). Wild-type and bre-5(yle17) animals were fed rhodamine-labeled Cry5B toxin for 1.5 hours and then imaged with DIC, in the rhodamine channel to visualize toxin, and in the FITC channel to visualize autofluorescent gut granules. Toxin was detected inside the wild-type gut cells and often colocalized with lysosomal gut granules. Toxin was not detected inside the bre-5 mutant gut cells but was confined to the lumen. Anterior is to the left in each panel; arrows point to the anterior end of the gut. Exposures are identical for each pair of wild-type and bre-5(yle17) images.
To rule out the possibility that the bre-5 mutant gut was generally defective in endocytosis, we fed wild-type and bre-5(ye17) animals the lipophilic dye FM4-64, a marker for membrane-mediated endocytosis, and rhodamine-labeled bovine serum albumin (BSA), a marker for fluid-phase endocytosis. Within 20 min, FM4-64 dye entered gut cells and colocalized with lysosomal gut granules in both wild-type and mutant animals, with indistinguishable kinetics. Rhodamine-BSA also entered wild-type and mutant gut cells with similar kinetics, but took much longer to detect than did FM4-64 or toxin. These data indicate that membrane-mediated and fluid-phase endocytosis occur relatively normally in the bre-5 mutant. Moreover, the finding that the rapid uptake of toxin into gut cells more closely resembles uptake of FM4-64 than that of rhodamine-BSA is consistent with toxin entering gut cells by membrane association rather than by fluid-phase endocytosis.

We have also ruled out the idea that bre-5 mutant animals have altered feeding behaviors that might affect the ability of toxin to interact with the gut membrane. We found that pharyngeal pumping rate (243 ± 9 pumps per min in wild type, 242 ± 11 in bre-5(ye17); n = 10 for both) and defecation rate (48 ± 5 s per cycle in wild type, 49 ± 7 s per cycle in bre-5(ye17); n = 10 for both) are not affected in the bre-5 mutant.

To address how widespread the bre-5 resistance mechanism might be, we tested whether bre-5 mutants were resistant to other Bt toxins. We took advantage of the fact that there is a known Bt toxin, Cry1Aa, that is part of the same phylogenetic subgroup of Bt toxins as Cry5B and is toxic to both nematodes and insects (2, 24, 25). Cry1Aa is 23% identical to Cry1Ac and 34% identical to Cry5B in the toxin domain. As with Cry5B, wild-type C. elegans fed Cry1Aa rapidly show gut damage. We found that bre-5(ye17) animals were sick on plates expressing high levels of Cry1Aa but were healthy on plates expressing lower levels of Cry1Aa that were still toxic to wild-type animals. To quantitate this dose-dependent resistance, we performed brood size assays for wild-type and bre-5(ye17) animals in the presence of variable amounts of Cry1Aa toxin (Fig. 3) (26). These data indicate that, relative to the wild type, bre-5(ye17) is 19 times as resistant to Cry1Aa.

Because bre-5 mutants show resistance to two divergent Bt toxins that share only 34% identity, this mechanism of resistance is likely to be applicable to other Bt toxins as well. Our Cry1Aa data also suggest that this mechanism is relevant for insects, because Cry1Aa is toxic to the beetle Diabrotica spp. It is likely that Cry1Aa recognizes the same carbohydrate structure in the beetle as in the nematode, and that bre-5-mediated resistance could develop in this insect with this toxin.

Our identification and characterization of bre-5 as a Bt resistance gene provides evidence in vivo for the importance of carbohydrates in Bt toxicity and the development of resistance. It is noteworthy that in the commercially important insecticidal toxins Cry1Aa and Cry3A, subtilisin-like aspartic proteinases, there is a carbohydrate structure in the beetle as well as in the nematode, and this structure in the beetle as well as in the nematode, and this structure is relevant for insects, because Cry1Aa is toxic to this insect with this toxin.

Our results potentially explain a dilemma in the Bt field, namely that a single toxin can bind to at least two receptors that are completely distinct. The Bt field has been concerned about how widespread this type of resistance is among different invertebrates—and how to deal with it—is vital for the long-term effectiveness of this important technology.

References and Notes

10. We injected bre-5(ye17) (6) hermaphrodites with a DNA cocktail containing the cosmid T12G3 (10 ng/µl) and plasmid pHF4 (80 ng/µl), which expresses a dominant rol-6 gene. Two of three stably transformed lines showed robust rescue. The 4.3-kb fragment was made by polymerase chain reaction (PCR) amplification with a mixture of Taq and Pfu polymerases using T12G3 and primers 5'-GAGCTGTCAGCAACCACTCTCAGCTTTG-3' and 5'-GATATGCAAAT TCGAT TCGT-GGCTCTAGACACCAACTCTC-3' and subcloning of the resulting 4.3-kb piece into pBluescript. When this plasmid was injected at 10 ng/µl along with pHF4 at 50 ng/µl, robust F1 and F2 rescue (2 of 2 lines) was seen. The ye107 allele was isolated in a genetic screen for animals resistant to Escherichia coli–expressed Cry5B (6); ye107 fails to complement bre-5(ye17) but complements alleles of the other four bre genes (L. Marroquin, R. V. Aroian, unpublished data).
11. For cDNA cloning, see supplementary material at Science Online (www.sciencemag.org/cgi/content/full/293/5531/860/DC1).
15. The subclone that encodes the entire bre-5 DNA cloned into pBluescript was PCR-amplified by T3 and T7 primers. RNA was prepared and purified from this PCR product using Megascript T3 and T7 kits (Ambion), annealed, and injected into wild-type adults. After 16 to 24 hours, L1- to L3-staged progeny were transferred to plates spread with E. coli expressing Cry5B toxin and scored 24 and 48 hours later for resistance. We have confirmed that injection of unrelated dsRNAs does not lead to resistance.
22. Cry5B crude spore lysate pellets (6) were resuspended with 3.8 ml of water and toxin crystals solubilized with 30 ml of acid solubilization buffer (8.7 mM
Myotonic Dystrophy Type 2 Caused by a CCTG Expansion in Intron 1 of ZNF9

Christina L. Liquori,1,2 Kenneth Ricker,4 Melinda L. Moseley,1,2 Jennifer F. Jacobsen,1,2 Wolfram Kress,5 Susan L. Naylor,6 John W. Day,1,3e Laura P. W. Ranum1,2a

Myotonic dystrophy (DM), the most common form of muscular dystrophy in adults, can be caused by a mutation on either chromosome 19q13 (DM1) or 3q21 (DM2/PROKM). DM1 is caused by a CTG expansion in the 3′ untranslated region of the dystrophia myotonica–protein kinase gene (DMPK). Several mechanisms have been invoked to explain how this mutation, which does not alter the protein–coding portion of a gene, causes the specific constellation of clinical features characteristic of DM. We now report that DM2 is caused by a CCTG expansion (mean ~5000 repeats) located in intron 1 of the zinc finger protein 9 (ZNF9) gene. Parallels between these mutations indicate that microsatellite expansions in RNA can be pathogenic and cause the multisystemic features of DM1 and DM2.

DM is a dominantly inherited, multisystemic disease with a consistent constellation of seemingly unrelated and rare clinical features including myotonia, muscular dystrophy, cardiac conduction defects, posterior irisred cataracts, and endocrine disorders (1). DM was first described nearly 100 years ago (2), but the existence of more than one genetic cause was only recognized after genetic testing became available for myotonic dystrophy type 1 (DM1) (3, 4).

DM1 is caused by an expanded CTG repeat on chromosome 19 that is both in the 3′ untranslated region of the dystrophia myotonica–protein kinase (DMPK) gene, and in the promoter region of the immediately adjacent homeodomain gene SIX5 (5, 6). How the CTG expansion in a noncoding region of a gene causes the complex DM phenotype remains unclear (5, 6). Suggested mechanisms include: (i) haplinsufficiency of the dystrophia myotonica–protein kinase (DMPK) protein (7); (ii) altered expression of neighboring genes, including SIX5 (8–12); and (iii) pathogenic effects of the CUG expansion in RNA which accumulates as nuclear foci (13, 14) and disrupts cellular function (15). We developed a model expressing CUG repeats in transgenic mice and found that a CUG expansion of 3.3 μg/ml wild-type animals had a brood of 5.1 ± 3.3 progeny (n = 20), whereas bre-5(ye17) animals had a brood of 99 ± 28 progeny (n = 20, compared to 104 ± 36 progeny without toxin).

DMPK knockout has cardiac abnormalities (20); and SIX5 knockouts have cataracts (21, 22). Taken together, these data have been interpreted to suggest that each theory may contribute to DM1 pathogenesis and that DM1 may be a regional gene disorder (5, 6).

Defining a second human mutation that causes the multisystemic effects of DM, and identifying what is common to these diseases at the molecular level, provides an independent means of determining the pathogenic pathway of DM. Toward this goal, we mapped the myotonic dystrophy type 2 (DM2/proximal myotonic myopathy (PROMM)) locus to chromosome 3q21 (23, 24) and have used position cloning to identify the DM2 mutation.

The DM2 region was narrowed to a 2-cM interval (25) by analyzing 10 recombinant chromosomes (25). Sequence data (26, 27) from this region, which is partially covered by 14 bacterial artificial chromosomes (BACs), was used to develop 80 short tandem repeat (STR) markers. Linkage disequilibrium analysis (27) was performed on 64 parent-offspring trios in which affected individuals had the clinical features of DM but not the DM1 mutation. Transmission disequilibrium testing (28) and analysis of conserved ancestral haplotypes narrowed the DM2 locus to a region of ~320 kilobases (kb) (Fig. 1A).

One of the markers in linkage disequilibrium with DM2, CL3N58 (P = 0.000001), showed an aberrant segregation pattern. All affected individuals appeared to be homozygous by polymerase chain reaction (PCR) (29), and affected children appeared not to inherit an allele from their affected parent (Fig. 1, B and C). Southern (DNA) analysis was performed (30) to investigate the possibility that the aberrant segregation pattern was caused by a repeat expansion or other rearrangement. In addition to the expected normal allele, we detected a variably sized expanded allele, too large to amplify by PCR, that was found only in affected individuals (Fig. 1, B and D). Modified electrophoresis conditions

1Institute of Human Genetics; 2Department of Genetics, Cell Biology, and Development; and 3Department of Neurology MMC 206, 420 Delaware Street SE, University of Minnesota, Minneapolis, MN 55455, USA. *Department of Neurology and Institute of Human Genetics, University of Würzburg, Germany. 4Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284, USA. 5To whom correspondence should be addressed. E-mail: johnday@umn.edu (J.W.D.); ranum001@umn.edu (L.P.W.R.).