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Monitoring of *Bacillus thuringiensis* Cry1Ac Resistance in *Helicoverpa armigera* (Hübner) (Noctuidae: Lepidoptera)

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Laboratory selection pressure of *Bacillus thuringiensis* Cry1Ac toxin against a selected strain of cotton bollworm (*Helicoverpa armigera*) increased the resistance to the toxin 83-fold. Sequence tagged microsatellite (STMS) markers were employed to genetically map the gene(s) for resistance to the *Bacillus thuringiensis* toxin. Out of the 43 STMS markers used, Ha 34 was found to be polymorphic between the two parental types and two bulks. Single factor analysis of variance using the marker locus genotypic class mean body weight values revealed strong linkage between the marker and trait ($P \leq 0.0001$). The resistant parent had two fragments while the susceptible had one fragment. The sequences of the resistant and susceptible samples with Ha 34 marker were done. The data showed that the lower band of resistant sample gave 479 bases and was submitted to the GenBank with Accession No. AJ875063. On the other hand, the susceptible sample contained 491 bases and its sequence was submitted to the GenBank with Accession No. AJ875064. This is the first molecular mapping of Cry1Ac resistance in the insect under study. This in turn will facilitate assessment, monitoring, and management of resistance in pest populations, and increase our understanding of Bt Cry1Ac resistance.

Introduction

The common soil bacterium *Bacillus thuringiensis* (*Bt*) produces crystals containing proteins toxic to certain insects, but harmless to most other organisms including humans, wildlife, and most beneficial insects (11). Transgenic crops with *Bt cry1Ac* gene proved highly effective against larval insect pests (12). *Bt* cotton is now cultivated in as many as 18 different countries over a total area of about 14 million acres (ca 5.7 x 10^6/ha). *Bt* cotton is effective against attack by the bollworm complex, although amongst them *H. armigera* has shown resistance in the laboratory to *Bt* toxins (2, 3, 13).

Bt modified crops have been developed that express sufficient levels of insecticidal proteins to protect them from attacks by major insect pests. When these varieties are cultivated on large areas, *Bt* toxins could produce selection pressure for development of resistance in the target insect pests, as happened with chemical insecticides. The potential development of insect resistance is the most significant threat to the sustainable use of transgenic plants.

The genetics of *Heliothis* and *Helicoverpa* resistance to chemical insecticides and to *Bt* have been studied (4, 5). A minor *Bt* resistance locus in a strain of *Heliothis virescens* exhibiting up to 10,000-fold resistance to Cry1Ac toxin was identified and mapped.It was also found that the major locus (*Bt R*-4) on linkage group 9 is responsible for about 80% of the total Cry1Ac resistance levels using RFLPs markers.

The microsatellite markers or SSR have been more widely used and are a powerful tool to differentiate between and within populations. The use of bulked segregate analysis (BSA) on pooled homozygous resistant and susceptible plant DNA has been reported to be an efficient strategy for finding molecular markers linked to a major dominant resistance gene in plants (8). The objective of the present study was to identify a marker linked to *Bt* Cry1Ac resistance in *H. armigera* using microsatellite markers by applying bulk segregant analysis method.

Materials and methods

A susceptible population of *H. armigera* was collected from a non *Bt*-cotton field and maintained on artificial diet under laboratory conditions (28° C ± 1° C, 70 to 80% RH, and 12:12 L:D regimes), while resistant larvae were collected from a *Bt*-cotton field and then exposed to selection pressure for one generation in the laboratory using 0.26 µg Cry1Ac toxin per gram of artificial diet. Ten grams of diet was mixed well with the Cry1Ac toxin using a pestle and mortar for each

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concentration, and divided into three groups, each of about 3 to 4 g, in 33-ml plastic cups. The Cry1Ac was prepared using the procedure described earlier (7, 6). The toxin concentration was determined by comparing absorbance with BSA standard curve obtained by a similar procedure (1) and the final toxin content was 40 µg/10 µl in the stock solution. Ten neonate *H. armigera* larvae were transferred 12 to 18 h after hatching into each cup using a fine brush. Six different concentrations; *viz*. 0.28, 1.12, 2.0, 2.5, 3.0 µg Cry1Ac toxin per gram of artificial diet, along with control (30 neonates in 3 replicates fed on normal diet) were used. Thirty neonates were used for each concentration with the three replicates. The observations were taken at 24 h intervals and the LC_{50} calculated after 96 h. Bioassay was conducted to determine LC_{50}s for both susceptible and resistant populations. The larvae were reared in the laboratory on chickpea-based semi synthetic diet (10) at a constant temperature of 28°C ± 1° C and relative humidity of 70 to 80%.

A cross was made using a male *H. armigera* showing 83-fold resistance and a susceptible female in a 60 ml plastic jar. The neonates of the F_1 generation were transferred onto normal artificial diet and allowed to feed until the pupal stage, and kept individually in 33-ml plastic cups until emergence. Twenty females were mated with 20 males of F_1 using wing character (greenish in color in case of male and brownish in case of female) in a single jar to obtain the F_2 population. Body weight was taken as a phenotypic character to express the extent of resistance quantitatively (4). DNA bulks were prepared

TABLE 1. SSRs marker with their product size.

Serial No.	Marker's name	Motif	Product Size (bp)	Marker sequence	
				F	R
1	Ha01	$(taactt)_3$	342	gcttcgaaaacgtggagttc	Ctcgatcaagcttttgaccc
2	Ha02	$(tacgac)_3$	470	acatcaggcctcaagaccac	Gtaggcgcctctgtcgtagt
3	Ha03	$(tgc)_6$	407	gctgtggagatgactgttgc	Gccgacttccaacaacaac
4	Ha04	$(ctacga)_3$	411	cactctgggaagggtcagtc	Acgatgctaagagtggcgat
5	Ha05	$(tgg)_7$	442	cgagttcgataccctcgttc	Aaaactatcgtcggcaccag
6	Ha06	$(acc)_6$	340	aacaccacagtcgtttcacg	Catccgttaccgtctccttg
7	Ha07	$(cga)_7$	424	gatctccgtcaacttgcaca	Acggtcacatgatgacaagg
8	Ha08	$(aac)_7$	451	aaacccagcgtgtttcagag	Acaccaaggcgtcgtaaaac
9	Ha09	$(gtc)_8$	321	ccacgatcacgtatcgaatg	atcaagatcgaagtcgcctg
10	Ha10	$(gtgc)_8$	326	ctcctgactgagcctttgct	acacacacacacacacgcac
11	Ha11	$(aaaccc)_3$	421	agagagcagtggtagcgagg	tggaacggttggtataggga
12	Ha12	$(attttt)_3$	467	cttggaaccacggacagaac	acgggctaaccacgtatgac
13	Ha13	$(tatag)_4$	408	gatgcttgagatctttccgc	tgagagttgctgggtaaggg
14	Ha14	$(taca)_5$	315	taggaagctgaagctggtgg	tacgggactggtttttcgag
15	Ha15	$(ca)_{10}$	412	agtggccaggatttatcacg	cattctctggagcttctcgg
16	HA16	$(cgcccg)_4$	396	ctgtggatgtccaagttccc	aatggggtgagttcgctaga
17	HA17	$(acatcg)_3$	399	agaagctttgcacccgttc	tcgatgtcgatgtcgatg
18	HA18	$(gccgc)_4$	397	ttaacctttggaccgctcac	cgtaccaactctgctccacc
19	HA19	$(at)_{15}$	421	gcgacactgctttggatttc	gggtcactaagttcggtaggc
20	HA20	$(tgg)_7$	442	cgagttcgataccctcgttc	aaaactatcgtcggcaccag
21	HA21	$(acc)_6$	340	aacaccacagtcgtttcacg	catccgttaccgtctccttg
22	HA22	$(at)_{12}$	475	ggaaccgatagaggccagat	gggaaacaaagggaggagag
23	HA23	$(at)_9$	479	tcgacaacctcaacgaacac	gagagggaccacgactaaagc
24	Ha24	$(gtt)_6$	313	cactcttcgtcatcgccc	gtagcggcacaaacgagg
25	Ha25	$(gttggt)_6$	325	cagggttgatgttgagggac	acgagcaccagcaaagagtt
26	Ha26	$(cca)_6$	476	gcgcgaacaaactaaaccag	aggcggtggtaacatttcag
27	Ha27	$(taaaaa)_3$	418	atgtttaccatgtgtccggc	gtccagaaaagtccatgcag
28	Ha28	$(ataa)_6$	413	aggacctttgcttgcgaac	gtaatcagtgccacacgctg
29	Ha29	$(ata)_6$	324	tgccctcacagaataaaggg	gtcattggttttgctgcc
30	Ha30	$(att)_6$	499	gttcggatcgctatttggg	agatgatagccaacaacggc
31	Ha31	$(ac)_{12}$	150	caccggaggataggaggagt	cacgtgtgggtgtgtgtgta
32	Ha32	$(ac)_8$	211	atgttatccgcatccgagtc	cgcgctatagagaccgtgac
33	Ha33	(at)13	165	gaggtcgttaaacgtctggtg	ccaacgttgacttaggtcgg
34	Ha34	$(tgc)_5$	315	gtttgttgtgggactcctga	tagcacatgcagcaaaccc
35	Ha35	$(act)_5$	388	gcggatctgcagagaaaaac	ggccagttgtcgtcgtagtag
36	Ha36	$(tat)_5$	493	atatgcctgtggatgatgcc	agttcaaggagcgcagatag
37	Ha37	$(att)_5$	318	atcttcgaacgtatcgctgc	actgatagattttgcggccc
38	Ha38	$(ggc)_5$	414	catggcacgtgttgttcttc	cagctcgtagctctgacacg
39	Ha39	$(taa)_5$	464	gttgaatgcacctttctccc	aaaggctctcgcattgtagg
40	Ha40	$(aca)_5$	362	ccgtgtgctgatggagtatg	cgtcactccttgtcactcca
41	Ha41	$(act)_5$	388	gcggatctgcagagaaaaac	ggccagttgtcgtcgtagtag
42	Ha42	$(gaa)_5$	474	cccttggtcgaggtacaaag	tgctcggaactctaactgacc
43	Ha43	$(cct)_5$	461	ttacagggtctggttccgtt	tgtgcatcgaaccttactcg

as described by Michelmore et al. (8). It was assumed that the larger larvae were resistant compared to the smaller larvae, which would be susceptible. Accordingly, 150 neonates (of the F_2 generation) were selected after hatching and exposed to artificial diet containing 0.035 µg Cry1Ac/g diet (sublethal dose as per our preliminary experiment) for 11 days to inhibit the growth. The body weight of 113 larvae were recorded in the F_2 population and classified into resistant and susceptible individuals. Ten resistance larvae of F_2 were selected for DNA bulk, as well as 10 susceptible larvae (having low body weight) were selected for susceptible DNA bulk. Each larva was placed separately in a 1.5 ml Eppendorff tube and kept at -80 °C until DNA extraction.

The microsatellite markers designed and developed for the first time in *silico* using the SSR tool (www.gramene.org/gramene/searches/ssrtool), and primer 3, from the available sequence information in the GenBank of *H. armigera,* and other related species. The list of microsatellite primers used in the present investigation is given in Table 1.

The DNA isolation method provided by Dr. David Heckel (personal communication) was used with slight modifications. DNA was quantified using the gel quantification method, in which the samples were loaded on 0.8% agarose gel in 1 X TAE buffer. Uncut genomic λ DNA was used as the standard. PCR was performed to test the accuracy of the quantification. A sample of 10 µl was used in each PCR reaction (9), and assessed within parent types, their bulk, as well as F_2 population. The PCR was performed in a total volume of 10 µl containing 25 to 40 ng templates. Template DNA was initially denatured at 94° C for 5 min followed by 35 cycles of PCR amplification. The amplified products were electrophoretically resolved on 1.5% agarose gel using 1 X TAE buffer at 70 V for 2.5 to 3 h. For better resolution the samples were finally resolved on 10% polyacrylamide gel for 4 to 5 h.

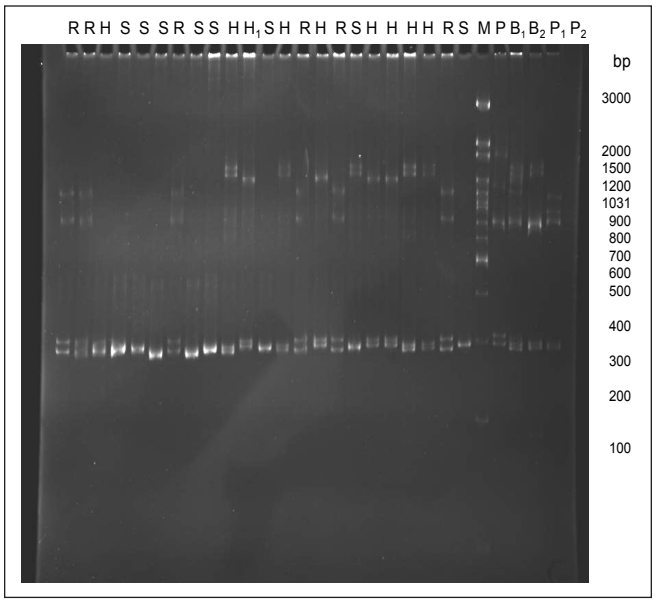

FIG. 1. Parental types with their bulks as well as individuals from F2 population. M: 100 bp DNA marker, P1: resistant parent, P2: susceptible parent, B1: bulk for resistant type, B2: bulk for susceptible type, S: susceptible type, R: resistant type, H1: heterozygote, and H: second heterozygote type.

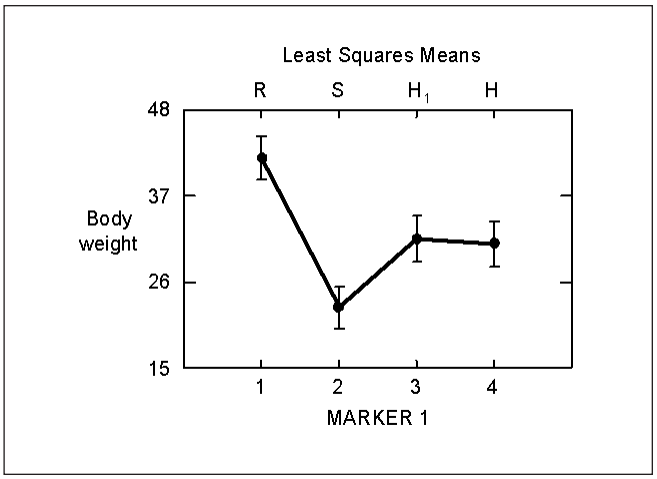

FIG. 2. Correlation between the phenotypic character (body weight) of F2 population and its four-genotypes (RR: A, SS: B, RS: H and H1 categories) with the Ha 34 microsatellite marker R: resistant type, S: susceptible type, H1: heterozygote type, and H: second heterozygote type.

Using the PCR-based sequencing method, the DNA from lower bands and DNA from upper bands of resistant lanes, as well as the single band of susceptible lanes identified, were eluted from 10% acrylamide gel and sent for sequencing. The association between the marker and body weight that was used to reflect *Bt* resistance was established by single factor analysis of variance using the statistical package SYSSTAT (version11, Richmond, California, USA).

Results and discussion

By comparing data from the resistant strain as well as the susceptible strain, our results showed that there was an 83-fold difference in resistance. The LC_{50} did not differ significantly between the reciprocal crosses

of the susceptible and resistant strains. This suggested that the mapping population from resistant male X susceptible female or resistant female X susceptible male crosses will not affect molecular mapping of the underlying genetic factor. Body weight was taken as a phenotypic parameter to distinguish the resistant larvae from susceptible ones. Since the Cry toxin is inhibiting the growth of the larvae, it was assumed that a larger larva is a resistant one when comparing with a smaller larva, which will be susceptible (4). The sequence of the Ha 34 microsatellite marker was submitted to the database GenBank under Accession No. BN000694.

Out of the 43 primers tested, the primer Ha 34 was able to distinguish the two parents as well as the two bulks, indicating its potential use as a linked marker to the trait. Single individual analysis of F_2 population was carried out with this marker to investigate the marker-trait association. Ha 34 marker amplified had two fragments (310 and 290 bp) with the resistant parent while the susceptible had one fragment (350). In the F_2 population these fragments gave four genotypes, namely R (310 + 290 bp fragments), S (350 bp), H (310 + 300 bp) and H_1 (300 + 290 bp). Out of 113 individuals, 29, 31, 27, and 26 individuals belonged to R, S, H, and H_1 classes, respectively. The resistant R class had a mean body weight of 41.89 mg when the susceptible S class had a mean body weight of 22.77 mg. The H and H_1 classes had 27 and 26 mg mean body weights, respectively. Of the 113 DNA samples of the F_2 population genotyped, there were four categories; *viz*. resistant P_1 type, susceptible P_2 type, and two types of heterozygotes, H type and H_1 type with the polymorphic microsatellite marker Ha 34 (Fig. 1). The differences between H and H_1 was not statistically significant, this suggested that the upper band in resistant individuals is responsible for inheritance of the resistant trait (Fig. 2).

Based on the bioassay results, we found that a single or a few genes control *Bt* Cry1Ac resistance in *H. armigera*. The analysis of data from the microsatellite marker linked to the Cry1Ac resistance trait, suggested that a single or a few genes are responsible for controlling *Bt* Cry1Ac resistance in *H. armigera.* The sequencing data showed that the lower band of the resistant sample had 479 bases and was submitted to the EMBL GenBank database with Accession No. AJ875063. On the other hand, the susceptible sample had 491 bases and this sequence was also submitted to the EMBL GenBank under Accession No. AJ875064.

Conclusion

It can be concluded that the microsatellite marker Ha 34 produced a high potential of polymorphism between resistant and susceptible individuals and their bulks. Once this marker co-segregates with resistance trait in R X S individuals, these would be helpful in determining the number of resistant, susceptible, and heterozygote individuals in the field. In addition to that, the possibility of monitoring the *Bt* resistance *cry1ac* gene(s) has become very easy at any field infested with *H. armigera* using Ha 34 microsatellite marker even without conducting any kind of bioassays, which may give imprecise information about the actual population and its biodiversity. This study will be helpful for further work to control the *Bt* Cry1Ac resistance in the insect under investigation. Thus, mapping *Bt* resistance in the insect under investigation will be helpful to isolate the complete gene responsible for the resistance.

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