

Bacillus thuringiensis Cry1Ac Resistance Frequency in Tobacco Budworm (Lepidoptera: Noctuidae)

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ABSTRACT The tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), is one of the most important pests of cotton, *Gossypium hirsutum* L., that has become resistant to a wide range of synthetic insecticides. Cry1Ac-expressing cotton has proven its effectiveness against this insect since its introduction in North America in 1996. However, the constant exposure of tobacco budworm to this protein toxin may result in the development of resistance to it. To estimate the frequency of alleles that confer resistance to a 1.0 µg of *Bacillus thuringiensis* Cry1Ac diagnostic concentration in field-collected insects, the second generation (F₂) of 1,001 single-pair families from seven geographical regions representing 2,202 alleles from natural populations was screened in 2006 and 2007 without finding major resistant alleles. Neonates of 56 single-pair families were able to develop to second instar on the diagnostic concentration in the initial screen, but only seven of these lines did so again in a second confirmatory screen. Minor resistance alleles to Cry1Ac may be quite common in natural populations of *H. virescens*. Our estimated resistance allele frequencies (0.0036–0.0263) were not significantly different from a previously published estimate from 1993. There is no evidence that *H. virescens* populations have become more resistant to Cry1Ac.

KEY WORDS *Heliothis virescens*, insecticide resistance management, F₂ screen, Bt-resistance allele frequency, single-pair families

During the first decade after the first commercial planting of genetically modified cotton, *Gossypium*

hirsutum L. (*Bacillus thuringiensis* Berliner [Bt] cotton, yr ≥1996) area planted with Bt cotton has grown ≥15 times, reaching 12.1 million ha around the world (ISAAA 2007). Because the Bt cottons constantly express the Cry1Ac protein from *B. thuringiensis*, the widespread and prolonged exposure to Bt proteins provides a constant selection pressure on the target pests, representing one of the largest selections for resistance development in insect populations the world has ever seen (Tabashnik et al. 2003). To prevent or delay the development of *B. thuringiensis* resistance in target pests, an insecticide resistance management strategy for Bt cotton is mandated by the U.S. Environmental Protection Agency. The effectiveness of this plan is based on the premise that transgenic plants express a “high dose” of the Bt protein(s) and that the implementation of a structured refuge of non Bt-expressing plants will mitigate the likelihood of resistance evolution (Matten and Reynolds 2003). This strategy is believed to have helped maintain the susceptibility of target pests such as the tobacco budworm, *Heliothis virescens* (F.), and the pink bollworm, *Pectinophora gossypiella* (Sauders), to Bt proteins since the introduction of Bt cotton (Tabashnik et al. 2006). Early detection of Bt resistance is important for the preservation of this effective agricultural biotech-

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nology as well as the evaluation of the effectiveness of resistance management strategies.

The tobacco budworm has acquired resistance to a wide range of synthetic insecticides (Sparks 1981, Luttrell et al. 1987, Hardee et al. 2001, Terán-Vargas et al. 2005). Before the commercial introduction of Bt cottons in 1995, very low frequencies of resistance to *B. thuringiensis* Cry1Ac were detected in field populations. Three Cry1Ac-resistant insects, with a cadherin gene-like mutation, were found in a sample of 1,025 field-collected tobacco budworm males, establishing this specific resistance frequency at 1.5×10^{-3} (Gould et al. 1997). Tobacco budworm samples collected between 1996 and 2002 were analyzed by Gahan et al. (2007) looking for a particular resistance mechanism by using molecular techniques showed no Cry1Ac resistant alleles in a large sample size ($n > 7,000$).

The goal of this study was to estimate a Cry1Ac resistance frequency that assessed all types of Cry1Ac resistance mechanisms by screening single-pair families from seven geographies of the North America Cotton Belt, a decade after the commercial introduction of Bt cottons.

Materials and Methods

Samples. Field-collected tobacco budworms were obtained at different times and from different plant hosts or pheromone traps from the following states: Arkansas, Louisiana, Mississippi, North Carolina, Tamaulipas (Mexico), and Texas. Garbanzo (*Cicer arietinum* L.), velvetleaf (*Abutilon theophrasti* Medikus), cotton, and tobacco (*Nicotiana tabacum* L.) plots were planted to obtain *H. virescens* eggs and larvae. Insects (except from Mississippi) were delivered to the USDA-ARS facility in Stoneville, MS, by overnight carrier, for testing.

Single-Pair Families. Field-collected larvae or pheromone trap-collected males (Mississippi and Texas only) were used to produce single-pair families. A single-pair family consisted of enclosing two *H. virescens* moths from a field-collection or a field-collected and a *B. thuringiensis*-susceptible moth of the USDA-ARS Stoneville, MS, colony. Pairs (parental P_0 generation) were held in 500-ml plastic containers (42505LY, Consolidated Plastic Co., Twinsburg, OH), with the top covered with Batist cloth (Zweigart, Piscataway, NJ), given free access to 10% sucrose solution in a plastic cup (37-ml [T-125, Solo, Urbana, IL]) with a paper tissue (Kleenex Kimberly-Clark, Roswell, GA) stuffed in it. Containers were placed in front of a window facing north with natural photoperiod, kept at $28 \pm 2^\circ\text{C}$ and $70 \pm 12\%$ RH. Sixty first-generation (F_1) neonates per single-pair family were placed on insect artificial diet (Blanco et al. 2008a) under the environmental conditions described above, except that they were not placed in front of a window. F_1 moths belonging to a single-pair family were sib-mated with six (± 1) pairs as described in Blanco et al. (2008b).

Cry1Ac Resistance Screening. Second generation (F_2) neonates were exposed to 1.0 μg of Cry1Ac (MVP II insecticide, Mycogen Corporation, San Di-

ego, CA) per ml of insect artificial diet that represents the upper fiducial limit for arresting larval development beyond first instar (molting inhibitory concentration [MIC]; Siegfried et al. 2000) ($\text{MIC}_{99} = 0.64 \mu\text{g}$, fiducial limits = 0.48–1.04; Blanco et al. 2007a) of the susceptible USDA-ARS Stoneville *H. virescens* colony. The treated insect artificial diet was dispensed (1.0 ± 0.15 ml per well) into bioassay trays (BAW-128, C-D International, Pitman, NJ). A single-pair family F_2 screening bioassay consisted of 96 wells containing the Cry1Ac diagnostic concentration and 32 cells containing control (0 μg of Cry1Ac) diet. Bioassays were kept under the previously described environmental conditions and evaluated 7 ± 1 d later by rating as “molting inhibition” criteria the number of dead and surviving first-instar larvae (molting inhibitory concentration). Any larvae of a single-pair family that developed to second instar or older on the diagnostic concentration triggered a confirmation process. The confirmation process was conducted by transferring all the survivors of the diagnostic concentration and all live larvae developing on control diet to freshly prepared control diet for moth emergence. This process consisted of retesting, using the methods previously described, at least one of the following crosses: 1) the F_3 offspring of sib-mated surviving F_2 moths that developed on the diagnostic concentration, expecting 100% homozygous resistant offspring; and/or 2) the F_3 offspring of sib-mating F_2 moths of that particular single-pair family that developed in control diet, expecting 0–100% homozygous resistant offspring; and/or 3) the second generation offspring (F_4) of backcrossing surviving F_2 moths of the retested family with ARS moths, expecting 0–25% homozygous resistant offspring. F_3 or F_4 neonates were exposed to the diagnostic concentration and control diet as previously described (the most commonly used method). If sufficient F_3 larvae were produced, they were also exposed to a serial dilution of 10 Cry1Ac concentrations (0, 0.06, 0.12, 0.20, 0.25, 0.40, 0.50, 0.75, 1.00, and 2.00 $\mu\text{g}/\text{ml}$ diet) to obtain the MIC_{50} .

Statistical Analyses. Data were analyzed using SAS Proc Probit Log Normal in SAS program (SAS Institute 2001) and differences in MIC_{50} values of single-pair families and the laboratory susceptible colony were considered significant if the 95% CL of the resistance ratio at the MIC_{50} level did not include 1.0 (Robertson and Priesler 1992).

R allele frequencies were estimated using Bayesian estimators. For locations with a mixture of single-parent and two-parent lines, we used equation 5 in Stodola et al. (2006). For locations with only single-parent lines screened, we used a simplification of equation 4 (Stodola et al. 2006) with $N_f = 0$. For single-parent lines, this equation simplified to

$$p_R = 1 - (1 - E[P])^{1/2}$$

where p_R is the estimated R allele frequency and $E[P]$ is the expected frequency of lines that test positive for resistance. For a uniform prior, $E[P] = (S + 1)/(N + 2)$, where S is the number of lines testing positive and N is the total number of lines tested.

Table 1. Origin, establishment and tests performed with single-pair *H. virescens* families collected in 2006 and 2007 screened for *B. thuringiensis* Cry1Ac protein susceptibility

Location	% origin of samples tested	P ₀ matings established/ matings attempted	Single-pair families tested	
			No.	Bioassays with >second instar larvae
Arkansas, Washington Co.	Garbanzo (100%)	19/112	9	2
Louisiana, Franklin Parish	Velvetleaf (100%)	188/583	124	6
Mississippi, Washington Co.	Garbanzo (90%), pheromone trapped males (10%)	241/687	182	5
North Carolina, Johnston Co.	Tobacco (100%)	262/566	157	20
Tamaulipas, Cuauhtemoc M.	Garbanzo (100%)	44/164	37	1
Texas, Brazos and Nueces Co.	Garbanzo (14%), Pheromone trapped males 86%	905/1,846	492	22
USDA-ARS Cry1Ac-susceptible colony	73/78	70	0	

Andow and Alstad (1998) provided a method for calculating the type 2 error rate for an F₂ screen when resistance alleles are rare, and designated this probability P_{No} . Minor corrections to these formulae were provided by Stodola and Andow (2004). Calculation of P_{No} requires an estimate of μ , F₂ larval mortality not related to the screen, and integral values of M , the number of males contributing to the F₂ generation, F , the number of females contributing to the F₂ generation, and J , the number of offspring per female screened in the F₂ generation. Nonscreen mortality μ was estimated from the control larvae for each F₂ line. The average M , F , and J were estimated for all the lines successfully screened. As these averages were not integral values, we estimated P_{No} for all eight combinations of the integral values bracketing the three averages and weighting these estimates to correspond to the average value for M , F , and J .

We also were interested in comparing our allele frequency estimates with previously published results (Gould et al. 1997) to see whether there has been an increase in R allele frequency. We used the joint likelihood ratio statistic (details in Wenes et al. 2006), $W(q_1, q_2)$, which is based on the estimated probability distributions of our estimate of the frequency of resistance alleles (denoted q_2) and the previously published estimate of R allele frequency (denoted q_1). We converted the Gould et al. (1997) results to a Bayesian estimate, which has a beta distribution. Using $W(q_1, q_2)$, we calculated the joint 95% credibility region around the estimated q_1 and q_2 . If the credibility region overlapped the hypothesis $q_1 = q_2$, then the sample estimates were not significantly different. The joint log-likelihood function was modified from that given in Wenes et al. (2006), taking into account the estimators used here (equation 5 from Stodola et al. 2006, and equation 1 above).

Labor and Cost Estimation. On randomly chosen days, information was gathered on the time involved performing different field or laboratory tasks. Cost of materials was obtained as 2006 and 2007 prices in Mississippi.

Results and Discussion

One-thousand and one single-pair families were tested for Cry1Ac susceptibility in 2006–2007 representing 2,202 field-collected alleles. Fifty-six single-pair families had at least one larva that developed beyond first instar (Table 1). By state, the numbers of single-pair families that had second instar larvae or older on the diagnostic concentration ranged from 2.7% of the samples (Mississippi and Tamaulipas) to 22.2% (Arkansas). Retesting was possible on 75% of these suspicious single-pair families (Table 2); the other 25% were lost, perhaps due to inbreeding depression.

There are several ways to calculate R allele frequencies. If all of the 56 families carried an allele for partial resistance, designated R_p , then an upper estimate of the frequency of partial resistance alleles is given in Table 3 by using the data from the initial screen. Because 25% of the lines could not be retested, the seven positive lines from the retest (Table 2) provide a lower estimate of the frequency of partial resistance (Table 3, second screen). Although our previous work (Blanco et al. 2008b) suggested that the rate of false positives from either test should be low, the fact that 31 of the 56 lines had only one larva developing to second instar indicated that the true frequency may be closer to the lower limit. For Arkansas, Louisiana, Mississippi, and Tamaulipas (TA), these two estimates of R_p were not statistically different (based on overlap of the 95% CIs). North Carolina and Texas had much larger sample sizes, and the estimates based on the initial and second screens were significantly different. Pooling all of the data, the expected frequency of partial resistance was estimated to be between 0.0036 and 0.0263 and may be closer to 0.0036 (Table 3).

We were unable to determine definitively that any of the resistant lines expressed a major gene for resistance. The most promising line from the second test, the Louisiana line with 15 second instars (Table 2), could not be established as a viable colony, in spite of our efforts of conducting the three confirmation crosses. If this line carried a major allele for resistance, designated R , then the estimated R allele frequencies

Table 2. Results of the bioassays of field-collected *H. virescens* in 2006 and 2007 screened with a 1.0 µg of CryIAc diagnostic concentration

Single-pair Origin (host)	Initial F ₂ screen test								Retest of the ≥F ₃ generation								MIC ₅₀ ^a	RR ^b	
	Larval development on control (0 µg) diet				Larval development on CryIAc (1.0 µg/ml) diet				Larval development on control (0 µg) diet				Larval development on CryIAc (1.0 µg/ml) diet						
	Dead	First	Second	Third	Dead	First	Second	Third	Dead	First	Second	Third	Dead	First	Second	Third			
AR (garbanzo)	1	0	5	26	60	33	2	0	1	0	0	0	31	90	6	0	0	N.A. ^c	N.A.
AR (garbanzo)	1	0	2	29	93	1	1	1	1	0	2	2	29	93	1	0	0	0.08	1.06
LA (garbanzo)	0	0	0	32	30	65	1	0	2	0	4	4	26	93	3	0	0	N.A.	N.A.
LA (velvetleaf)	2	0	5	25	46	47	2	0					Single-pair family lost						
LA (velvetleaf)	9	0	1	21	80	15	1	0	2	0	1	1	29	86	10	0	0	N.A.	N.A.
LA (velvetleaf)	0	0	1	29	84	12	1	0					Single-pair family lost						
LA (velvetleaf)	1	4	6	21	56	37	2	0	1	0	0	0	31	92	3	1	0	N.A.	N.A.
LA (velvetleaf)	4	5	5	18	89	5	2	0	0	0	0	0	32	9	38	15	0	N.A.	N.A.
MS (garbanzo)	0	3	1	27	81	12	1	0	1	0	3	3	28	88	8	0	0	0.05	0.47
MS (garbanzo)	0	0	1	31	46	49	1	0	1	0	3	3	28	90	6	0	0	N.A.	N.A.
MS (garbanzo)	3	5	2	22	58	37	1	0	0	1	2	2	12	44	4	0	0	N.A.	N.A.
MS (pheromone)	0	0	3	29	31	63	2	0	0	0	1	1	31	82	14	0	0	N.A.	N.A.
MS (pheromone)	1	0	4	27	75	15	1	0					Single-pair family lost						
NC (tobacco)	2	3	0	27	58	21	1	0	1	1	0	0	30	88	8	0	0	N.A.	N.A.
NC (tobacco)	1	0	2	29	53	33	10	0					Single-pair family lost						
NC (tobacco)	2	0	1	29	78	16	18	0	4	2	2	2	24	95	1	0	0	N.A.	N.A.
NC (tobacco)	1	0	0	31	92	3	1	0	0	2	0	0	30	89	5	0	0	0.12	1.88
NC (tobacco)	3	8	6	14	73	20	3	0	1	1	3	3	25	88	7	0	0	0.004	0.06
NC (tobacco)	1	0	4	11	37	10	1	0	1	0	2	2	29	71	9	0	0	N.A.	N.A.
NC (tobacco)	6	0	15	11	70	25	1	0	1	0	1	1	14	77	3	0	0	0.04	0.43
NC (tobacco)	1	1	0	30	61	33	2	0					Single-pair family lost						
NC (tobacco)	1	1	4	26	78	10	8	0					Single-pair family lost						
NC (tobacco)	3	1	1	27	82	13	1	0	0	0	2	2	30	79	16	0	0	N.A.	N.A.
NC (tobacco)	3	1	2	26	85	9	2	0	1	0	3	3	27	91	5	0	0	N.A.	N.A.
NC (tobacco)	0	0	0	16	42	3	3	0					Single-pair family lost						
NC (tobacco)	0	0	1	30	91	4	1	0	0	0	0	0	32	77	18	1	0	N.A.	N.A.
NC (tobacco)	5	2	1	24	88	7	1	0	0	1	2	2	13	46	2	0	0	N.A.	N.A.
NC (tobacco)	0	0	8	24	77	18	1	0					Single-pair family lost						
NC (tobacco)	2	0	2	28	92	2	2	0	1	0	0	0	31	72	22	1	0	0.058	0.57
NC (tobacco)	0	0	4	28	29	61	5	0					Single-pair family lost						
NC (tobacco)	0	0	1	31	79	16	1	0	0	0	1	1	31	88	8	0	0	N.A.	N.A.
NC (tobacco)	0	0	8	24	54	41	1	0	2	1	3	3	26	69	11	0	0	N.A.	N.A.
NC (tobacco)	1	3	5	21	34	54	2	0	0	0	4	4	28	93	3	0	0	N.A.	N.A.
TA (garbanzo)	2	2	6	22	72	21	3	0	0	0	1	1	31	87	9	0	0	N.A.	N.A.
TX (pheromone)	5	1	3	23	26	5	17	0	0	0	2	2	30	44	4	0	0	0.088	1.22
TX (pheromone)	3	2	6	69	49	2	43	2	1	1	1	1	29	95	1	0	0	0.101	1.4
TX (pheromone)	0	4	5	23	80	15	1	0	0	0	0	0	31	88	5	0	0	N.A.	N.A.
TX (pheromone)	1	0	0	15	26	21	1	0					Single-pair family lost						
TX (pheromone)	1	0	6	25	53	41	1	0	0	2	2	2	12	43	5	0	0	N.A.	N.A.
TX (pheromone)	3	5	8	16	20	72	4	0	2	1	4	4	25	90	4	0	0	N.A.	N.A.
TX (pheromone)	2	1	0	28	31	60	1	0	2	0	2	2	28	69	24	1	0	N.A.	N.A.
TX (cotton)	1	1	6	24	50	43	2	0					Single-pair family lost						
TX (pheromone)	2	0	0	30	95	0	1	0	0	0	0	0	32	76	4	0	0	N.A.	N.A.
TX (pheromone)	0	3	2	27	89	6	1	0					Single-pair family lost						
TX (pheromone)	0	2	2	28	36	59	1	0	3	2	3	3	21	96	0	0	0	N.A.	N.A.
TX (pheromone)	1	0	1	30	91	4	1	0	0	0	4	4	27	77	16	0	0	N.A.	N.A.
TX (pheromone)	4	1	5	22	64	31	1	0					Single-pair family lost						
TX (pheromone)	2	1	2	27	85	10	1	0	1	1	1	1	29	82	13	0	0	N.A.	N.A.
TX (pheromone)	0	1	2	29	79	15	1	0	0	1	2	2	13	76	4	0	0	N.A.	N.A.
TX (pheromone)	3	2	2	25	49	44	2	0	4	3	6	6	19	93	3	0	0	N.A.	N.A.
TX (pheromone)	2	1	4	25	95	0	1	0					Single-pair family lost						
TX (pheromone)	8	3	0	21	82	13	1	0	4	4	2	2	22	76	4	0	0	0.02	0.15
TX (pheromone)	2	0	2	28	69	22	5	0	2	2	3	3	25	77	17	2	0	0.03	0.19
TX (pheromone)	1	1	8	22	76	18	2	0	0	0	2	2	30	89	7	0	0	0.03	0.21
TX (garbanzo)	5	5	2	20	62	32	2	0	1	0	3	3	12	66	14	0	0	0.02	0.13
TX (pheromone)	0	2	0	30	69	22	5	0	0	1	0	0	31	53	42	1	0	0.05	0.30

^a Molting inhibitory concentration.

^b Resistance ratio; *, RR significantly different ($P < 0.05$) from the susceptible (ARS) colony.

^c Not applicable.

are given in Table 4 (second screening columns). If the line did not carry a major allele, the estimated *R* allele frequencies are also given in Table 4 (zero columns). Based on the overlap of the 95% CIs, the two estimates did not differ significantly for any of the sites or the pooled estimate. The expected *R* allele frequency for the pooled data are either 0.0004 or 0.0009 (depending on whether no lines were considered resistant or only one line was considered resistant), and the combined 95% CI is (0.0000, 0.0025).

We were also interested to determine whether our estimated *R* allele frequency was different from that

previously reported by Gould et al. (1997)), because a higher estimate could have indicated that resistance was evolving. We found that a Bayesian estimator for the Gould et al. (1997) rangewide data were 0.0019 with a 95% CI of (0.0005, 0.0043). Our rangewide estimate was either 0.0004 or 0.0009 with a 95% CI of (0.0000, 0.0025). Using the likelihood function, the probability that these two estimates were different was either 0.054 or 0.166 (both n.s. at $\alpha = 0.05$). Thus, there has been no significant change in *R* allele frequency in *H. virescens* between 1993 and 2007. This supports, but does not prove, the suggestion that the

Table 3. Partial resistance allele frequency (Bayesian estimates) from *H. virescens* single-pair families screened for *B. thuringiensis* Cry1Ac resistance

Location	No. lines screened			No. potential positives (all single parent lines)		Estimated Rp allele frequency						Sign
	Total	2-Parent	1-Parent	Initial screening	Second screening	Upper estimate			Lower estimate			
						Expected (initial)	Lower 95% CI	Upper 95% CI	Expected (second)	Lower 95% CI	Upper 95% CI	
AR	9	0	9	2	0	0.1472	0.0339	0.3337	0.0465	0	0.1391	ns
LA	124	37	87	6	2	0.0218	0.0089	0.0407	0.0093	0.0019	0.0223	ns
MS	182	30	152	5	0	0.0142	0.0052	0.0275	0.0023	0	0.0070	ns
NC	157	10	147	20	2	0.0646	0.0406	0.0941	0.0089	0.0019	0.0214	*
TX	492	23	469	22	3	0.0225	0.0144	0.0325	0.0039	0.0011	0.0085	*
TA	37	0	37	1	0	0.0260	0.0032	0.0716	0.0129	0	0.0387	ns
Pooled	1,001	100	901	56	7	0.0263	0.0200	0.0335	0.0036	0.0016	0.0065	*

*Asterisk represents.

Bt cotton insect resistance management strategy has been effective at delaying the evolution of resistance in *H. virescens*.

The possibility that the F₂ screen underestimated the R allele frequency is related to the statistical possibility that a line with resistance was not identified because the allele was lost from the line, and no RR homozygotes were produced, or RR homozygotes died before they could be identified. Nonscreen mortality μ was estimated from the 26,977 control larvae to be 5.99%. The average *M*, *F* and *J* values were 6.03, 6.03, and 6.03 for both 2-parent and single-parent families. The estimated P_{No} was 0.123, which means that there was about a one-eighths probability that the screen missed detecting at least one resistance allele. The probability that two resistance alleles were missed in the entire experiment was <1/64, which is small. Even if we had missed detecting one resistance allele, the estimated resistance allele frequency has not changed significantly since 1993.

It is possible that false positives were observed in the initial screening. One possible reason is that a mistake could have been made while producing the diagnostic concentration in the diet. The fact that nearly all of the single-pair families that were simultaneously tested had no larvae developing beyond first instar indicated that this was unlikely. Several of the single-pair families were backcrossed with ARS moths for retesting and to propagate the line, but many of these families were lost (Table 2) due to infertility.

When the lines were sib-mated again, the females copulated (spermatophores were found in their bursa copulatrix), but produced nonfertile eggs. Inbreeding depression interacting with other unknown factors may have been responsible for this infertility, and is being further investigated.

Another possibility explaining why some lines had development into second instars might be variation in intrinsic susceptibility to Cry1Ac. The MIC₅₀ of the second generation (F₂) neonates from a cross between a field-collected P₀ female and a laboratory P₀ male (ARS colony) was 47 ± 7.5% (mean ± SEM) higher than in F₂ neonates of two ARS parents. The F₂ MIC₅₀ of the cross between an ARS P₀ female moth and a field-collected P₀ male was 23 ± 2.8% higher, and the F₂ MIC₅₀ of the cross between two field-collected P₀ moths was 36 ± 11.3% higher than the response of the F₂ neonates of two ARS parents. This indicates that a slightly, but significantly, lower susceptibility to Cry1Ac is achieved possibly due to the hybrid vigor obtained from these field crosses. Therefore, development beyond first instar on the 1.0 µg/ml of Cry1Ac diagnostic concentration is possible. Although this diagnostic concentration may allow for some development of field-collected tobacco budworm families beyond the first instar, triggering an unnecessary confirmation process, it is a sensitive concentration that allows detection of minor or partial resistant alleles. Because it has been the diagnostic concentration

Table 4. Major resistance allele frequency (Bayesian estimates) from *H. virescens* single-pair families screened for *B. thuringiensis* Cry1Ac resistance

Location	No. lines screened			No. potential positives (all single parent lines)		Estimated R allele frequency						
	Total	2-Parent	1-Parent	Second screening	Zero	Second screening estimate			Zero estimate			
						Expected	Lower 95% CI	Upper 95% CI	Expected	Lower 95% CI	Upper 95% CI	
AR	9	0	9	0	0	0.0465	0	0.1391	0.0465	0	0.1391	ns
LA	124	37	87	1	0	0.0061	0.0007	0.0171	0.0031	0	0.0092	ns
MS	182	30	152	0	0	0.0023	0	0.007	0.0023	0	0.007	ns
NC	157	10	147	0	0	0.003	0	0.0089	0.003	0	0.0089	ns
TX	492	23	469	0	0	0.001	0	0.0029	0.001	0	0.0029	ns
TA	37	0	37	0	0	0.0129	0	0.0387	0.0129	0	0.0387	ns
Pooled	1,001	100	901	1	0	0.0009	0.0001	0.0025	0.0004	0	0.0014	ns

used in the past in our monitoring program (Blanco et al. 2005), it also allows for comparisons between years.

The field-collected single-parent F_2 screen method used here has its advantages and disadvantages (Stodola et al. 2006). For example, it facilitates the evaluation of more field-collected alleles, even though it reduces in half the number of alleles that can be screened per F_2 family compared with two field-collected parent lines. Copulations between two field-collected tobacco budworm moths that produced viable F_1 progeny under our laboratory conditions is only 17–30% successful, depending on the time of the year. Also, the method allows for the use of surpluses of one sex collected at pheromone traps, on host plants, or at light traps. Obtaining *H. virescens* in the field on certain host plants may occur during only a few weeks of the year (Blanco et al. 2007b), and the sex ratio may be biased on some collection days.

Some of the costs and labor needed for this type of testing can be high but it is possible to modify the procedure to reduce expenditures. For example, the maintenance of a *B. thuringiensis*-susceptible reference colony is the most labor intensive and costly endeavor of this work. Part of this cost can be reduced if, for example, tobacco budworm was purchased from a commercial vendor. One *H. virescens* pupa from the USDA-ARS Stoneville facility has a price of \$0.05. Setting-up and maintaining single-pair families involves also intensive work and cost. A skilled worker can set up 10 single-pair families per hour. The cost of mating containers (\$0.77) and supplies to rear 60 F_1 larvae (diet = \$1.26, \$1.80 cups + lids) can be reduced if the items are used multiple times, and if diet is produced locally. This can result in a $\geq 300\%$ cost savings compared with the purchase of diet and insects from a commercial vendor. Reducing the number of F_1 larvae to only 30 per single-pair family is another way of cutting costs. The cost of each bioassay test was \$8.75 for materials and \$0.22 for locally-prepared diet. This amount can be slightly reduced by reusing the bioassay trays. A skilled worker can set up 4.5 bioassay trays per hour. The total cost per line tested was \$120.00. As we progressed during this 2-yr study, we were able to use materials and labor more efficiently, lowering costs and improving the efficiency of laboratory personnel. Cost estimates for this type of work are described by Andow and Ives (2002) for *Ostrinia nubilalis* (Hübner).

The results of this study, through the use of bioassays capable of detecting most of the Cry1Ac resistance mechanisms, establish a more robust Bt resistance allele frequency in natural *H. virescens* populations than previous reports, because we screened all detectable Cry1Ac resistance mechanisms. It will be possible in the future to continue to monitor resistance frequency changes in subsequent samples.

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