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Cross-pollination in seed-blended refuge and selection for Vip3A resistance in a lepidopteran pest as detected by genomic monitoring

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The evolution of pest resistance to management tools reduces productivity and results in economic losses in agricultural systems. To slow its emergence and spread, monitoring and prevention practices are implemented in resistance management programs. Recent work suggests that genomic approaches can identify signs of emerging resistance to aid in resistance management. Here, we empirically examined the sensitivity of genomic monitoring for resistance management in transgenic Bt crops, a globally important agricultural innovation. Whole genome resequencing of wild North American *Helicoverpa zea* collected from non-expressing refuge and plants expressing Cry1Ab confirmed that resistance-associated signatures of selection were detectable after a single generation of exposure. Upon demonstrating its sensitivity, we applied genomic monitoring to wild *H. zea* that survived Vip3A exposure resulting from cross-pollination of refuge plants in seed-blended plots. Refuge seed interplanted with transgenic seed exposed *H. zea* to sublethal doses of Vip3A protein in corn ears and was associated with allele frequency divergence across the genome. Some of the greatest allele frequency divergence occurred in genomic regions adjacent to a previously described candidate gene for Vip3A resistance. Our work highlights the power of genomic monitoring to sensitively detect heritable changes associated with field exposure to Bt toxins and suggests that seed-blended refuge will likely hasten the evolution of resistance to Vip3A in lepidopteran pests.

Helicoverpa zea | genomic monitoring | *Bacillus thuringiensis* | pesticide resistance | polygenic adaptation

Significance

Pesticide resistance evolution is common in agricultural systems and results in reduced productivity and economic losses. Delaying the emergence of resistance and sensitively detecting it when it arises provides opportunities to prevent these losses and improve the durability of pest management innovations. Genomic approaches have the power to detect molecular signals of emerging resistance prior to crop failure, but there is a need to determine their sensitivity and develop best practices for their use in resistance management. Our work demonstrated that genomic monitoring could detect resistance-associated allele frequency divergence in a pest population after a single generation of exposure, and we detected signals of emerging resistance to Vip3A toxin.

Modern agricultural systems rely on large-scale pest suppression measures to prevent crop losses by insect, weed, and microbial pests (1–3). Evolution of pest resistance to these suppression measures presents an ever-increasing challenge, particularly as the emergence of resistance outpaces pesticide innovation (4), and growers bear billions of dollars in increased management costs (5, 6). Over the past four decades, the field of pesticide resistance management was developed to address this challenge (7). Its goals are to improve the durability of pest management tools by preventing, delaying, monitoring for, and mitigating emerging resistance in pests and pathogens prior to management failures.

Genetically engineered crops that express genes derived from the bacterium *Bacillus thuringiensis* (Bt) throughout their tissues represent an agricultural innovation that has played an important role in the management of insect pests on a global scale (8, 9). Target lepidopteran and coleopteran pests ingest the insecticidal proteins encoded by these genes while feeding on plant tissues, resulting in stunted growth and mortality. Early Bt crop cultivars expressed only a single crystalline (Cry) toxin, but current Bt crops express multiple Cry toxins, as well as vegetative insecticidal proteins (Vip) to suppress pest populations. Past and projected economic and environmental benefits of pest suppression with Bt crops were significant [e.g., (9–17)], and therefore, resistance management was prioritized on a global scale (17, 18). Early implementation of Bt resistance management, which included both prevention and monitoring strategies, undoubtedly slowed the evolution and spread of Bt resistance in target pests. Yet resistance evolved in some pests for which prevention and monitoring strategies fell short (8, 9, 19).

Bt resistance management has relied heavily on the high dose/refuge strategy, where a high dose cultivar producing 25 times the concentration of toxin required to kill susceptible individuals is planted alongside unprotected refuge plants (e.g., plants not expressing insecticidal toxins) (20–23). When resistance is monogenic and recessive, and especially when individuals bearing resistance alleles incur a fitness cost in the absence of toxin exposure (reviewed in ref. 22), refuge plants should maintain or promote increases in Bt susceptible genotypes in an insect population. Such refuge plants might include the same crop, other crops, or wild plant hosts that do not express Bt toxins. Susceptible individuals emerging from unprotected refuge should be much more numerous than resistant

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homozygotes emerging from the Bt varieties because of the low initial frequency of resistance-conferring alleles (22). Matings between rare resistant and numerous susceptible individuals will produce heterozygous offspring with low fitness on Bt-expressing plants, thereby slowing the spread of resistance alleles.

Unprotected refuge may be planted in a separate block adjacent to Bt-expressing crops (i.e., structured or pure stand refuge), but this has economic and operational limitations (24, 25). More recently, Bt corn has been made commercially available as a seed blend, where non-Bt seed is mixed with transgenic Bt seed and planted as a single block [i.e., a refuge in a bag; (24)]. Although this eliminates some of the known economic and operational limitations, it results in many adjacent Bt-expressing and refuge plants within the same field. Previous work suggested that movement of pest larvae between Bt-expressing and refuge plants can reduce the efficacy of seed-blended refuge (26–28). Moreover, for wind-pollinated crops like corn, adjacency provides opportunities for refuge pollination by Bt-expressing plants and subsequent kernel expression of Cry toxins. Kernels from a single ear of a non-Bt corn plant show mosaic Cry expression if they are fertilized by pollen from nearby Cry-expressing plants (29, 30). This mosaic expression resulting from cross-pollination effectively diminishes refuge area and allows for sublethal toxin exposure (24, 29, 31), making its utility for resistance prevention unclear. Diminished refuge area and sublethal toxin exposure would undermine the high dose/refuge strategy and likely hasten the emergence and spread of resistance in pests (32, 33).

Monitoring and identifying pest populations with early warning signs of resistance has also posed challenges, in part due to unclear thresholds for what constitutes resistance in some pest species. Measurements of change in resistance phenotypes can be highly variable due to confounding environmental variation. This makes establishment of thresholds for classifying a pest population as resistant challenging. In the case of Bt resistance management, early documents from the US-EPA advocated for monitoring to mitigate risk of resistance evolution, yet also expressed concerns about how to monitor and how to use monitoring data to trigger remedial action (18, 34). It was unclear, for example, whether remedial action should be triggered based on small changes in pest resistance phenotypes (18). Since the commercialization of Bt-expressing crops, monitoring protocols have evolved from simple dose/mortality and dose/development assays [e.g., (35, 36)] to estimation of Bt resistance allele frequencies using F1 and F2 progeny testing [e.g., (37–39)] and molecular diagnostics [e.g., (40)]. All of these approaches have strengths and weaknesses and have been used across the globe to monitor target lepidopteran and coleopteran pests for Bt resistance. Despite these efforts, a lack of clear criteria for triggering regulatory responses to genetically based Bt resistance in insects has contributed to field-evolved resistance to Bt toxins in eight major agricultural pests worldwide (8, 41, 42).

Helicoverpa zea is one costly North American crop pest that has evolved damaging levels of Bt resistance, despite long-term monitoring (43). Resistance evolution in *H. zea* was anticipated because the multi-toxin Bt cotton and corn cultivars introduced into agricultural production systems over the past 20 y do not result in a high dose for *H. zea* (36), making it an exception to the high dose/refuge resistance prevention strategy. To date, Cry1A and Cry2A resistant *H. zea* are found throughout North America (41–47). We have used emergence of Bt resistance in *H. zea* as a model to study both how genetic changes of small to moderate effect size accumulate in wild pest populations, leading to widespread resistance (48), and to determine whether knowledge of these changes can be used for resistance management.

Recently, there have been calls for the incorporation of modern genomics approaches into resistance monitoring programs (7, 46). Genomic DNA is transmitted from parent to offspring during reproduction, and therefore, resistance-conferring alleles encoded in DNA sequences are heritable. Genomic monitoring may be sensitive enough to detect early resistance-associated allele frequency changes on short time scales, signaling growing resistance. If successful, genomic monitoring would then reveal heritable changes in susceptibility and trigger remedial action before resistance reaches levels that cause widespread crop failure. In a previous study, we retrospectively applied genomic monitoring to archived samples of *H. zea* and identified genetic signals of Cry1Ab and Cry1A.105+Cry2Ab2 resistance 4 y prior to documentation of Bt crop failure (48), but we also noted that further development of genomic monitoring would require an efficient strategy to link signals of genomic change to resistance traits in wild insect populations.

Here, we examine the power of paired larval collections from Bt-expressing and refuge plants to document resistance and genomic signals of selection within a single generation in wild *H. zea*. Paired plots should produce resistance allele frequency differentials when a heterogeneous population is exposed to both Bt-expressing and refuge plants at a single point in time. If resistance-associated genomic variation exists within a population, Bt-expressing plants should select against susceptible individuals, resulting in genomic divergence between sequenced individuals originating from Bt-expressing and refuge plants. No a priori knowledge of a resistance mechanism is needed because allele frequencies are measured across the genome with whole genome resequencing data. Paired collections also provide evidence of exposure and fitness for individuals sampled directly from plants with known expression, allowing for phenotypes to be linked to genomic differences among groups.

A recent multi-state study measured pupal weight and survivorship of wild *H. zea* fed upon transgenic Bt corn pyramids expressing different combinations of Cry1Ab, Cry1F, and Vip3Aa20 (hereafter Vip3A), as well as non-Bt refuge corn plants, to document fitness effects following toxin exposure (49). We leveraged wild *H. zea* collected from one of these study sites to test whether genomic monitoring could detect significant resistance allele frequency divergence due to Cry toxin exposure in a single generation. A comparison of samples with Cry exposure to those collected from pure stand refuge corn revealed significant genomic divergence in a known Cry resistance–associated genomic region, demonstrating the sensitivity of genomic approaches for resistance monitoring.

After documenting the feasibility of our approach, we tested for genomic signals of selection by Vip3A in wild *H. zea*. Vip3A is the only toxin in commercially planted Bt pyramids with strong efficacy against Cry-resistant *H. zea* (50, 51), yet concerns about its durability are growing following reports of subtle changes in tolerance in wild *H. zea* populations (49, 52, 53) and increasing plant injury in the field (42, 54, 55). Moreover, recent studies have documented reduced survival and weight of *H. zea* collected from refuge plants blended with Vip3A-expressing plants relative to those from pure stand refuge, suggesting sublethal Vip3A toxin exposure in seed-blended refuge (49, 56). We demonstrated that putatively non-Bt refuge corn adjacent to Vip3A-expressing corn expressed toxin in their kernels, pointing to one possible mechanism for this sublethal exposure. Genomic comparisons of *H. zea* with and without Vip3A exposure revealed genome-wide changes, including a selective sweep encompassing a candidate gene known to bind Vip3A toxins in other insects. Our work demonstrates a drawback of seed-blended refuge for Bt corn, where cross-pollination

leads to mosaic kernel expression of Vip3A in refuge ears. Moreover, low-dose exposure to Vip3A toxins is associated with unique signatures of genomic selection, suggesting that seed-blended refuge could hasten the evolution of resistance in *H. zea*.

Results

Pupal Productivity of Wild *H. zea* Indicates Vip3A Exposure in Seed-Blended Plots. *H. zea* infested ears from non-Bt, two-toxin (Cry1Ab+Cry1F), and three-toxin (Cry1Ab+Cry1F+Vip3A) pyramided field corn were harvested from replicated plantings (n = 4) at the North Carolina State University Vernon James Research and Extension Center in Plymouth, North Carolina (NC) in 2019 and 2020. These plantings were part of a larger, multi-state study that paired toxin-expressing corn with non-expressing refuge corn in replicated pure stand and seed-blended plots (Fig. 1 *A–D* and *SI Appendix*, Table S1) to quantify the lethal and sublethal impacts of Bt on wild *H. zea* (49). A total of 955 pupae and pre-pupae emerged from freshly harvested corn ears collected in NC over the 2-y study period. We analyzed infestation rates and pupal weight from this subset of samples collected by Pezzini et al. (49) to document site-specific Bt exposure and site-specific resistance phenotypes, thereby allowing us to link them to signatures of Bt selection with genomic monitoring.

Susceptible wild *H. zea* should be unable to feed on Bt-expressing kernel tissue, resulting in lower infestation rates of ears from the two- and three-toxin pyramids relative to non-expressing corn. The number of pupae collected per 20 ears differed according to the number of toxins expressed in corn plants and whether ears were collected from pure stand or seed-blended plots (F = 18.02, df = 6, P < 0.001; Fig. 1*E*). Two-toxin pyramided corn generally produced fewer pupae than refuge corn, although differences were not statistically significant for either pure stand or seed-blended plots. There was a significant difference between three-toxin Bt and non-Bt plants in seed-blended plots (Fig. 1*E*), consistent with previous multi-state findings (49). Only three pupae (two live, one dead) were produced by ears of Vip3A expressing corn in our three toxin seed-blended plots, and no pupae were produced by ears from our pure stand of three-toxin expressing corn. A modest number of pupae were produced from non-Bt refuge planted among three-toxin pyramided plants (Fig. 1*E*), which is consistent with exposure to toxin-expressing rather than non-expressing plants.

Pupal weight, which reflects the ability of *H. zea* larvae to feed upon and convert kernel tissue to biomass before exiting a corn ear, also differed across treatments (F = 14.14, df = 6, P < 0.001; Fig. 1*F*). Weights were highest in pupae collected from pure stands of non-Bt refuge, followed by those collected from two-toxin pyramided corn in pure stands, and non-Bt refuge and two-toxin corn planted in seed-blended plots. Subtle decreases in pupal weight associated with exposure and resistance to Bt have been documented for this species (57), including in the multi-state survey by Pezzini et al. (49). *H. zea* collected in NC from non-Bt refuge blended with three-toxin pyramided corn plants had significantly lower pupal weight than those from pure stand refuge (Fig. 1*F*). Patterns of low pupal production and weight in *H. zea* collected from refuge corn within three-toxin expressing seed-blended plots, were both consistent with toxin exposure. Cry1Ab and Cry1F expressed in our two-toxin pyramids showed poor activity, leaving only Vip3A as capable of suppressing *H. zea* populations in three-toxin pyramids (Fig. 1*E*). Notably, while Vip3A

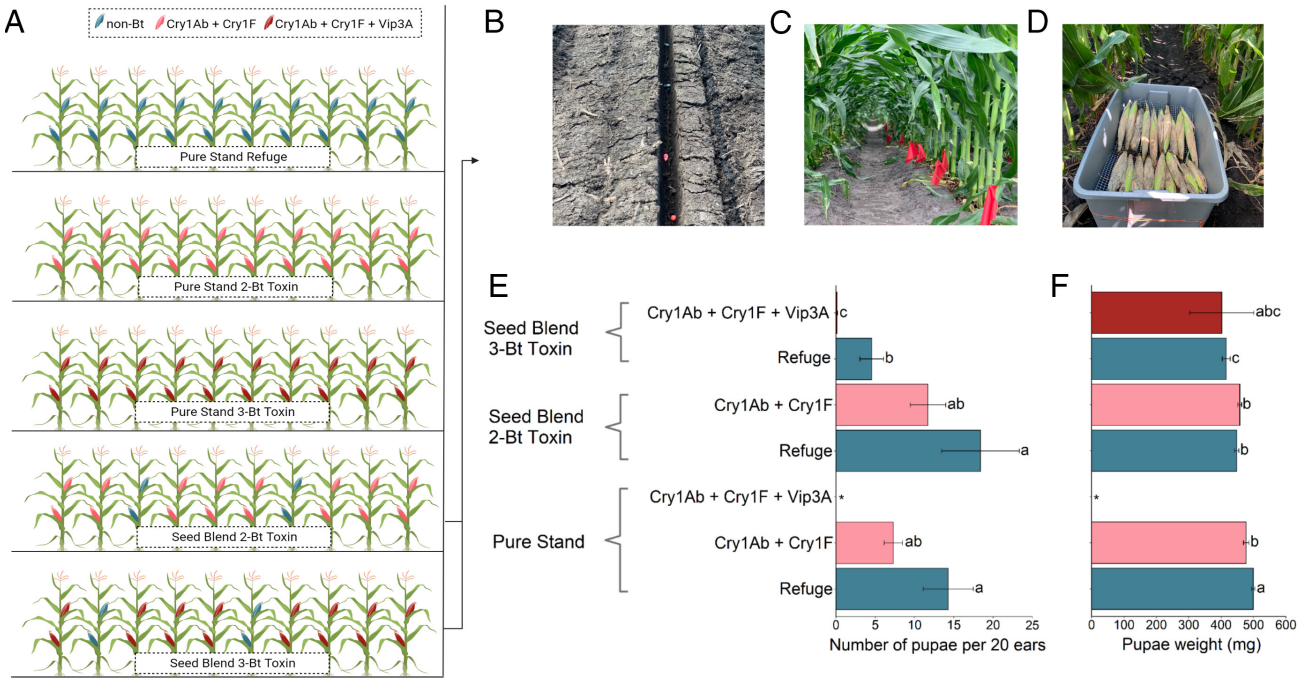

Fig. 1. Infestation rates and pupal weights of *H. zea* collected from experimental plots used to monitor for emerging Bt toxin resistance. (*A*) Non-Bt (refuge), Cry1Ab+Cry1F-expressing, and Cry1Ab+Cry1F+Vip3A-expressing field corn was planted in replicated pure stand or seed-blended plots (80% Bt plants and 20% non-Bt plants). (*B*) At planting, the color of conventional and Bt-expressing seed differed, which allowed for location of each plant type in seed-blended plots. (*C*) Bt-expressing plants in seed-blended plots were flagged. (*D*) Ears from each treatment were harvested and placed into pupation boxes. (*E*) *H. zea* that survived to complete larval development in each treatment were collected from the soil in each pupation box. Bars represent mean pupal counts (±SEM) out of 20 sampled ears per treatment and plot type for samples collected in North Carolina. (*F*) Pupal weights were measured and compared across treatments. Bars represent mean pupal weights (±SEM) per treatment and plot type for samples collected in North Carolina. Means with the same letters are not significantly different at α = 0.05 using Tukey–Kramer-adjusted pairwise comparisons of least square means. An asterisk (*) indicates no pupae were collected for that treatment. Panels *E* and *F* show a reanalyzed subset of data from a large multi-state survey by Pezzini et al. (49). Created with BioRender.com.

activity in three-toxin pyramided corn remains strong against *H. zea*, reduced infestation of non-Bt corn planted among three toxin pyramids, as well as the reduced weights of pupae produced by these ears signaled sublethal exposure to Vip3A at our NC study site, likely due to cross-pollination.

Contaminant Expression in Conventional Corn Plantings Adjacent to Vip3A Plantings. Kernel expression of Cry toxins has been linked to cross-pollination (29, 30) and has been proposed as one possible mechanism for sublethal Vip3A exposure (49). While attempts exist (55), to our knowledge, there is no published test that documents contaminant Vip3A expression due to cross-pollination. To test whether *H. zea* infesting non-Bt refuge plants adjacent to Vip3A-expressing plants might be exposed to toxin, we used a widely available protein detection kit (QuickStix, EnviroLogix) to measure Vip3A expression in kernels sampled from adjacent four row plantings of a Vip3A-expressing field corn cultivar and non-Bt field corn at the Central Maryland Research and Education Center (CMREC) in Beltsville, MD. Of 10 non-Bt ears planted furthest from the Vip3A expressing cultivar, 7 tested positive for Vip3A expression, while all 10 non-Bt ears planted adjacent to toxin-expressing corn were positive (Fig. 2 *A* and *B*). With our pooled approach, a positive test from non-Bt plants indicated that at least one of 20 kernels sampled per ear was pollinated by Vip3A-expressing corn and could express the toxin. As expected, all ears of the Vip3A-expressing cultivar tested positive for Vip expression (Fig. 2 *C* and *D*).

We also measured brightness of the test band relative to the control band to obtain a semi-quantitative measure of Vip3A expression. Vip3A test bands were darkest for the ears from expressing plants, and lighter for the ears from the non-Bt plants ($F = 38.36$, $df = 3$, $P = 2.63 \times 10^{-11}$; Fig. 2*E*), confirming higher expression in Vip3A-expressing ears. Pairwise post hoc t-tests showed that kernels from non-Bt corn planted furthest from the Vip3A-expressing plot had significantly lighter bands than all other treatments ($P = 8.4 \times 10^{-7}$, 1.6×10^{-10}, 4.3×10^{-10}; Fig. 2*E*). Vip3A test bands from non-Bt ears sampled closest to Vip3A-expressing ears were lighter than or equal to the test band brightness of Vip3A-expressing ears ($P = 0.035$, $P = 0.084$, Fig. 2*E*). qPCR (58) to validate QuickStix protein expression results revealed that Vip3A DNA was present in refuge kernels, albeit at lower copy numbers than for Vip3A-expressing plants (*SI Appendix*, Fig. S1 and Table S2; also see *SI Appendix, Supplemental Results*). These findings suggested that contaminant Vip3A expression is more likely to occur in non-Bt corn planted directly adjacent to Vip3A-expressing plants, and the level of Vip3A expression in kernels from non-Bt plants adjacent to Vip3A-expressing plants is high.

Population Genomic Analysis Reveals Little Genome-Wide Structure among Field-Collected *H. zea*. Field-evolved resistance to Cry toxins and their causal genomic regions have previously been documented (48). Moreover, evidence for Vip3A resistance alleles in wild *H. zea* was also recently reported (52). In light of these developments, we used Illumina whole genome sequencing (WGS) of wild *H. zea* collected from the seven treatments at our NC study site to determine whether genomic monitoring of insects collected from paired Bt-expressing and non-Bt plots could reveal a detectable increase in resistance allele frequencies in a single generation of exposure.

After quality filtering of our WGS dataset, 9.5 billion reads from 151 male and female *H. zea* were used for genomic analysis (*SI Appendix*, Table S1). Of these 151 individuals, 148 were collected in 2019, but 3 individuals sequenced for our Vip3A-exposed group were collected in 2020. We included these to increase our statistical power to detect signatures of Vip3A selection. Following filter-trimming and read alignment to a chromosome-scale *H. zea* assembly (accession PRJNA767434; mean read coverage depth = 18.8 ± 5.9 SD), variant calling produced 5,630,888 single nucleotide polymorphisms (SNPs) for downstream analyses (*SI Appendix, Supplemental Results*).

Major patterns of population structure were explored in the genome wide SNP data using a principal component analysis (PCA; *SI Appendix*, Fig. S2). Most individuals clustered into a single group, regardless of the cultivar- or plot-type they were sampled from. Only four individuals collected from non-Bt refuge

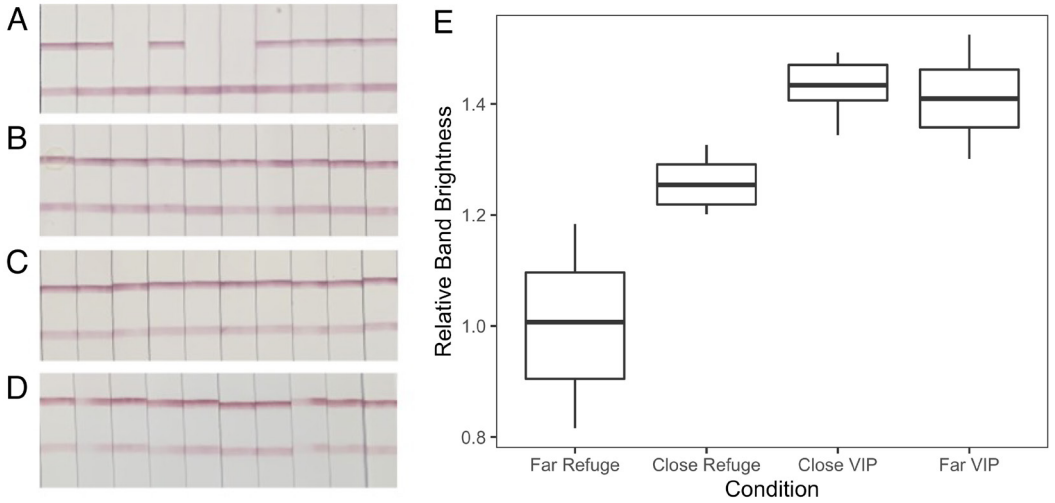

Fig. 2. Vip3A expression in adjacent Bt-expressing and non-expressing (pure stand refuge) field corn plots. QuickStix test results for (*A*) Vip3A expression in far refuge, (*B*) close refuge, (*C*) close Vip, and (*D*) far Vip. For 20 pooled kernels collected from each of 10 replicate ears per treatment, only three far refuge pools tested negative for Vip3A expression, while all other pools (including all close planted refuge) tested positive. The relative brightness of the test band (*Upper*; Vip3A) compared to the control bands (*Lower*) was greater for the Vip3A expressing corn than the conventional refuge corn pollinated by Vip3A expressing corn. Panel (*E*) depicts relative test band brightness from Vip3A expression tests for 20 pooled kernels from each of 10 replicate ears per treatment. Relative band brightness was calculated as control band brightness divided by the test band brightness. On average the Vip3A test band was darkest for close and far planted Vip3A corn, and was lightest for far planted refuge corn, with close planted refuge having an intermediate intensity. Differences in relative band brightness were statistically significant according to ANOVA ($F = 38.36$, $df = 3$, $P = 2.63 \times 10^{-11}$).

corn were found outside of this cluster, and each principal component (PC1 and PC2) explained 6% or less of the overall variation in our genomic dataset. This lack of population genomic structure was further supported by a genome-wide F_{ST} analysis conducted between individuals from different treatment:plot-type combinations (Table 1). We observed low genome-wide weighted F_{ST} values when individuals collected from pure stands of refuge corn, those least likely to express any toxins, were compared to individuals collected from two-toxin and three-toxin expressing corn and the likely cross-pollinated refuge ears in seed-blended plots. This minimal genome-wide population differentiation was to be expected because all individuals used in pairwise comparisons were collected from the same small sampling region (1.2 acres), and all but 3 Vip3A-exposed individuals were from the same generation in the same year. Despite the small geographic area sampled, there was little evidence of relatedness among individuals sequenced (*SI Appendix*, *Supplemental Results* and Fig. S3).

Genomic Signatures of Selection Following One Generation of Exposure to Cry-Expressing Corn. While there was no genome-wide differentiation among *H. zea* samples, we hypothesized that selection by Cry toxins could have resulted in significant F_{ST} values for local genomic regions associated with Cry resistance. We used Weir and Cockerham's (59) sliding windowed F_{ST} to identify the genomic windows with the strongest signals of divergence between individuals exposed to pure stand refuge and Cry-expressing corn. Our Cry-exposed group included samples collected from non-Bt corn planted as refuge in our two-toxin seed-blended plots, because Cry kernel expression due to cross-pollination was previously documented (29, 30). Sliding window averaged F_{ST} values were calculated for a range of window sizes, but here we report results from a 40-kb window with a 1-kb step size as results were consistent across window/step sizes (*SI Appendix*, Fig. S4). Divergence greater than the z-transformed F_{ST} (zF_{ST}) significance threshold of 6 was considered to be statistically significant, and zF_{ST} significance thresholds were calculated separately for the autosomes and Z chromosome as in Perrier et al. (60). Overall, there were 25 genomic windows where divergence between individuals exposed to non-Bt plants in pure stand and to stands including Cry-expressing corn exceeded our threshold for statistical significance (*SI Appendix*, Table S3). Allele frequency divergence in these windows could be associated with selection by Cry1Ab, which is known to have a polygenic basis for resistance (48), or Cry1F toxins, both of which are expressed in the two toxin pyramids. The genomic regions associated with field evolved Cry1F resistance have not been identified, but recent work has linked Cry1Ab resistance in wild *H. zea* to multiple regions of chromosome (Chr) 9 (48). All further proof-of-concept Cry resistance analyses focused on this chromosome because its role in field evolved Cry1Ab resistance is known.

Two windows of significant genomic divergence were detected between 5 and 6 Mb (peaks at 5.14 to 5.21 and 5.81 to 5.87 Mb) on Chr 9. These corresponded well to a region of significant temporal allele frequency change (5.74 to 5.79 Mb) in wild *H. zea,* which occurred concurrently with increasing adoption of Cry-expressing corn and cotton in North America (48). The full 5- to 6-Mb region was examined for candidate genes with potential to confer Cry1Ab resistance (*SI Appendix*, Table S4). Multiple genes could be identified within this 5- to 6-Mb region, including a cluster of trypsins found between 5.27 and 5.35 Mb. Changes in trypsin gene expression (61, 62) and midgut proteolytic activity (63–65) are associated with Cry1A and Cry2A toxin processing in Lepidoptera and can lead to resistance (66). Interestingly, trypsin activity and expression differed between laboratory-selected, Cry-resistant, and Cry-susceptible populations of *H. zea* in previous studies (64, 67). To test for midgut expression of these trypsins, we dissected and pooled midguts of laboratory-reared susceptible fifth instar *H. zea* for bulk RNA-sequencing (*SI Appendix*, *Supplemental Methods*). All seven of these trypsins were expressed in the *H. zea* midgut (*SI Appendix*, Fig. S5), demonstrating their potential for interaction with ingested Cry1Ab toxin (Fig. 3).

Pesticides are known to exert strong selection on their target organisms, and we used SNP frequency data from this resistance-associated region of Chr 9 to quantify the strength of selection (s) imposed by field exposure to Cry1Ab within a single generation. Due to widespread Cry1Ab resistance in *H. zea*, our calculations assumed that selection was acting against the minor allele (q) present in our field populations and that Cry1Ab resistance was either additive, an incompletely recessive trait as in previous studies (68, 69), or completely recessive. Ranges of s represent the selection intensity that would have been required to produce each of the SNP frequency differences across the most diverged 40-Kb window on Chr 9. When resistance conferred by the major allele (p) was additive, s ranged from 0.59 to 1. Incompletely recessive or completely recessive resistance required s-values ranging from 0.42 to 0.95 and 0.40 to 0.90, respectively, to produce the observed SNP frequency differences in this Cry resistance-associated genomic region (*SI Appendix*, Fig. S6). Assuming additivity or partial recessiveness of resistance increased the strength of selection required to cause the observed changes in SNP frequency, because resistance did not completely depend on having two copies of p. Strong selection against q would therefore have been required to further reduce its frequency. The values of s reported from our work are among the highest documented in field studies (70), but in line with those previously calculated from insecticide tolerance phenotypes (71).

Selection for Vip3A Resistance. Successful detection of significant allele frequency divergence following one generation of Cry1Ab selection in a Cry resistance-associated genomic region motivated us to scan the genomes of our wild *H. zea* for Vip3A resistance-associated genomic changes. We compared samples collected from Vip3A-expressing and Vip3A cross-pollinated corn with samples collected from non-Bt refuge corn in pure stands using a Weir and Cockerham's windowed F_{ST} as described above (Fig. 4*A*

Table 1. Genome-wide analysis of Weir and Cockerham's weighted F_{ST} as calculated with a 40-kb/1-kb window/step

		Weighted F_{ST}	
Pairwise comparison	Effect of treatment on pupal weight (mg)	Genome-wide mean	Window range
Pure stand non-Bt refuge v. *2-toxin exposed*	35.38	0.000033	(−0.013, 0.057)
Pure stand non-Bt refuge v. *3-toxin exposed*	98.26	0.000167	(−0.035, 0.151)

Pure stand non-Bt refuge indicates that *H. zea* samples were collected from corn plants derived from multiple rows of non-expressing seed planted adjacent to one another. Two-toxin or three-toxin exposed indicates that *H. zea* samples were collected directly from Bt-expressing plants or from refuge corn planted among Bt-expressing plants. The reference for comparison of all treatments (*ital*) are samples derived from refuge corn planted in a pure stand because they would have the lowest Bt kernel expression.

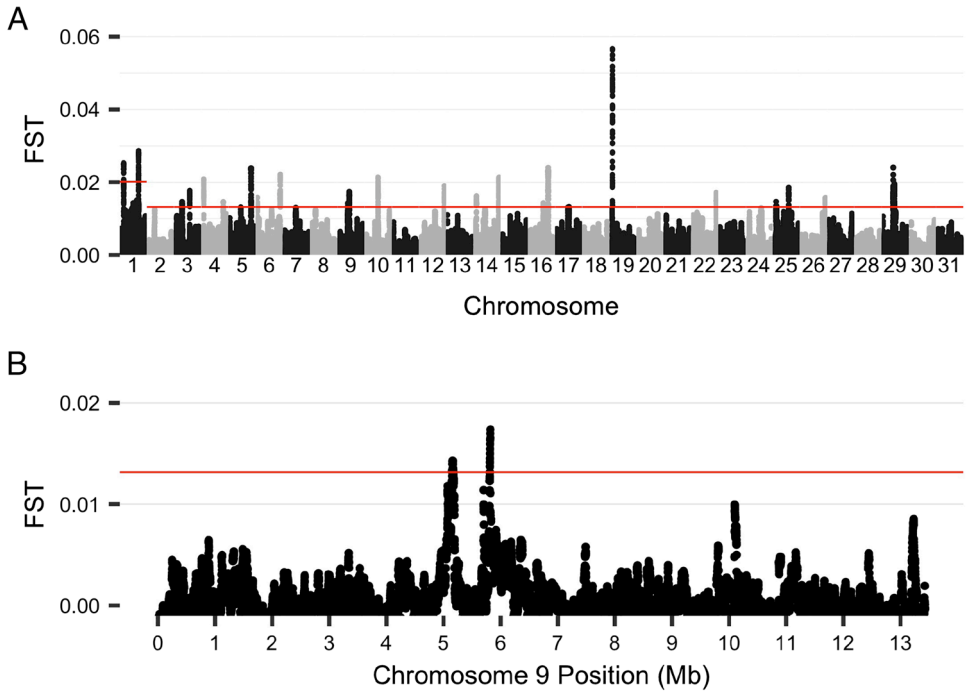

Fig. 3. Genomic divergence between *H. zea* collected from pure stand non-Bt refuge and *H. zea* with Cry toxin exposure. Each point represents divergence as measured by Weir and Cockerham's windowed F_{ST} in a 40-kb window sliding along the chromosome with a 1-kb step. Red lines indicate the significance threshold of $zF_{ST} > 6$. (*A*) Divergence across all 31 *H. zea* chromosomes. Chromosomes are plotted in alternating gray and black. (*B*) Divergence on Chr 9, a chromosome known to be associated with Cry1Ab resistance.

and *SI Appendix*, Fig. S7). Significant F_{ST} windows that were overlapping or directly adjacent to one another were merged to identify genomic regions under selection by Vip3A. This resulted in 19 windows (size range = 44 to 152 kb) of significantly elevated F_{ST} found on chromosomes 1, 3, 4, 6, 7, 12, 13, 14, 21, 22, 23, 24, 27, 28, and 31 (Table 2). These top windows do not overlap with Cry resistance-associated windows, suggesting that selection by Vip3A is being detected, rather than selection by Cry toxins in the pyramid (*SI Appendix*, Fig. S8).

The top three candidate windows were selected based upon high maximum F_{ST} and were located on Chr 1 from 14,976,001 to 15,057,000, Chr 12 from 1,246,001 to 1,324,000, and Chr 13 from 996,001 to 1,079,000. We further characterized signals of Vip3A selection at these windows by measuring both linkage disequilibrium (LD) in *H. zea* with Vip3A exposure (Fig. 4 *B–D*) and genetic distance between our samples (Fig. 4 *E–G*). Strong selection for resistance should elevate LD in these genomic regions in the Vip3A-exposed population and should reduce genetic distance among Vip3A-exposed individuals, relative to those collected from non-expressing pure stand refuge. Candidate windows on Chr 12 and 13 showed the expected patterns of LD that were characteristic of a selective sweep, but LD was weak within the region of genomic divergence on Chr 1.

Analysis of genetic distance between Vip3A-exposed individuals, and those from pure stand non-Bt refuge showed the strongest clustering in the Chr 12 window. Yet SNP variation failed to separate Vip3A-exposed individuals from those developed on pure stand refuge corn in all three candidate windows. This may result from selection on standing genetic variation, where mutations that already exist in a population become adaptive under new environmental conditions (72), and modest selection intensity resulting from low dose exposure allows a substantial fraction of the population to survive (73). This would increase the frequency of resistance mutations with modest effect size, but also the frequencies of multiple SNPs surrounding the resistance mutation, all of which are older than the selection pressure itself (72). Under these circumstances, multiple haplotypes contain the resistance-conferring mutation, and some SNPs from these resistance-conferring haplotypes are also shared by susceptible haplotypes. We postulate that this may be the case in our study. Most Vip3A-exposed individuals likely experienced a lower and varied dose of Vip3A because they are from non-Bt ears cross-pollinated by three toxin pyramids, which likely have mosaic kernel expression.

We examined regions of Chr 1, 12, and 13 showing elevated divergence for potential candidate genes associated with Vip3A resistance. Complete lists of lifted over gene annotations found within 100 kb of Vip-associated peaks on these chromosomes are in *SI Appendix*, Tables S5–S7. On Chr 1, we identified a gene annotated as papilin-like, which acts as a protease inhibitor. The candidate window on Chr 12 contained a gene annotated as Spartan-like, a metalloendopeptidase which typically functions to catalyze hydrolysis of peptide bonds. No candidate genes could be identified in the window on Chr 13 from 996,001 to 1,079,000 with the strongest F_{ST} signal. Yet less than 2 kb from the Chr 13 3,695,001 to 3,737,000 significant window and still within the region of elevated F_{ST} is tenascin-like, a gene likely homologous with an identified Vip3A binding protein in *Agrotis ipsilon* (74). We analyzed protein-coding changes within this putative candidate gene, and both the results of this analysis (*SI Appendix*, *Supplemental Results* and Fig. S9) as well as the location of the divergence peak upstream of tenascin-like suggest that if tenascin-like is under selection by Vip3A toxin, regulatory rather than protein coding changes are likely to be involved. Interestingly, this candidate window is also ca. 100 kb from another Vip resistance candidate gene, ribosomal protein S2 (75).

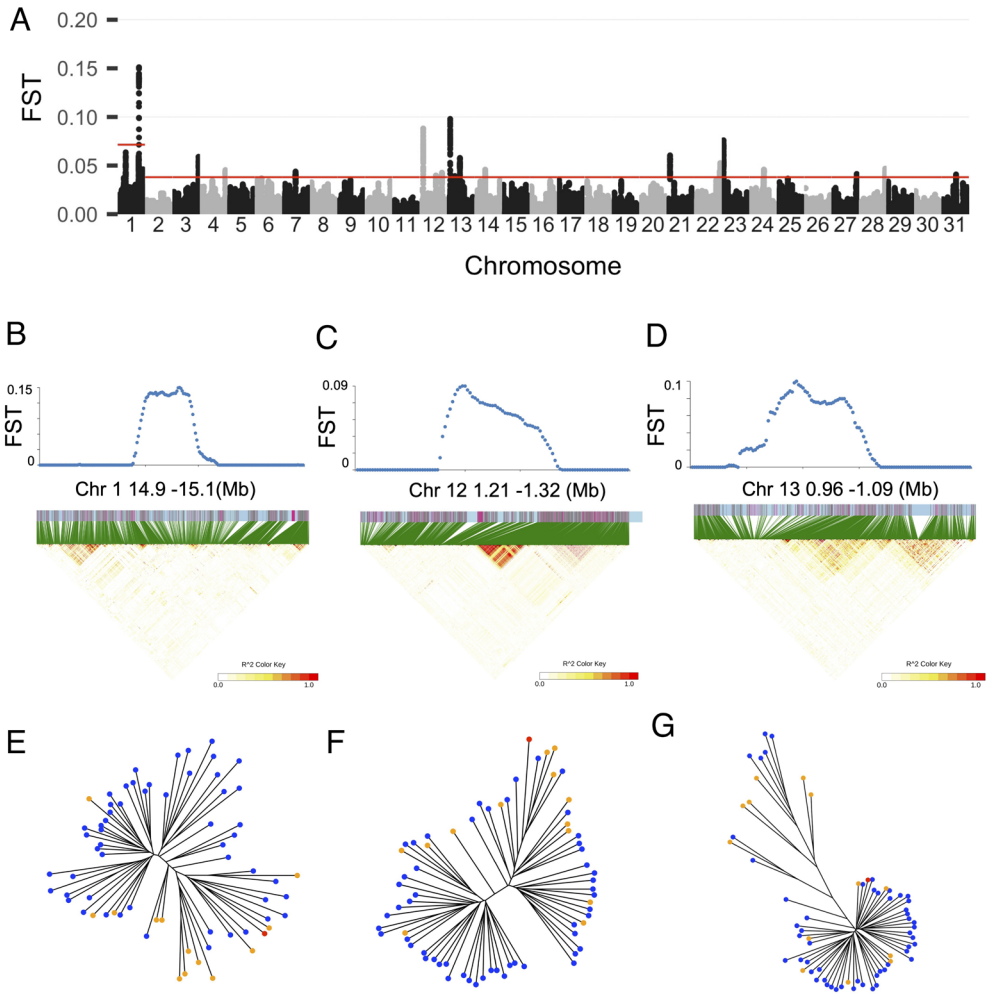

Fig. 4. Vip3A exposure-associated genome evolution. (*A*) Whole genomic divergence between *H. zea* collected from non-expressing structured refuge and Vip3A-expressing corn. Divergence was measured by Weir and Cockerham's sliding windowed F_{ST} across 40-kb windows with a 1-kb step. The red line indicates $zF_{ST} > 6$. For the top three candidate windows, max F_{ST} is plotted above r^2 heatmaps for Vip3A-exposed individuals for the peak regions (*B*) Chr 1 from 14,976,001 to 15,057,000, (*C*) Chr 12 from 1,246,001 to 1,324,000, and (*D*) Chr 13 from 996,001 to 1,079,000. Neighbor-joining SNP distance phylogenies are shown with structured refuge individual tips colored blue, individuals potentially Vip3A-exposed by cross-pollination of refuge corn colored yellow, and the one individual collected from Vip3A-expressing plant colored red. (*E*) Chr 1 peak, (*F*) Chr 12 peak, and (*G*) Chr 13 peak.

In or near the windows of elevated F_{ST} on other chromosomes, we also identified several genes with other functions potentially related to Bt resistance including a protease inhibitor (Chr 1), an alkaline phosphatase (Chr 3), immune-related genes (Chr 22), MAPK signaling (Chr 22), and transport proteins expressed in the midgut of insects (Chr 1). They did not contain genes specifically identified as candidates for Vip3A resistance including *myb*, scavenger receptor class C-like, or fibroblast growth factor receptors (76–78). These significant windows also contain poorly annotated and non-candidate genes, which potentially could also underlie the signals of Vip3A selection detected here. Future studies of Vip3A mode of action could shed further light on the genomic signatures of Vip3A selection reported here and provide insight into the efficacy of genomic monitoring.

Discussion

Our work addresses two challenges for pesticide resistance monitoring programs, using Bt resistance management in *H. zea* as a model. These include 1) the lack of a monitoring approach with potential to eliminate ambiguity regarding resistance-related remediation thresholds, and 2) limited empirical data assessing the phenotypic and genotypic impact of seed-blended refuges for resistance management. Current and past resistance monitoring strategies have focused on tracking changes in resistance phenotypes in wild pest populations or tracking allele frequency changes at known resistance genes. While these approaches have both strengths and weaknesses, they lack the power to identify subtle, heritable changes anywhere in the genome that would allow for detection of emerging resistance, particularly by previously undiscovered and multi-genic mechanisms (19).

To address this first challenge, we sequenced the genomes of wild *H. zea* collected from pure stand refuge and compared them to sequenced genomes of *H. zea* exposed to Cry1Ab and Cry1F. Our previous work demonstrated that a genome-scanning approach had the power to detect allele frequency changes in populations of wild *H. zea* collected over time with increasing Cry toxin exposure in the landscape (48). Yet determining which evolutionary responses were likely to be associated with Cry1Ab exposure, as opposed to selection by other environmental factors required execution of a separate experiment. Here, we adopted an experimental framework that applied genome scanning to samples collected from paired plots as a way to link a resistance phenotype (i.e., growth when feeding on toxin-expressing plants) to differences in allele

Table 2. Genomic regions of divergence following one generation of Vip3A exposure

Chr	Window start	Window end	Window size	Max F_{ST}	Mean F_{ST}
1	***14,976,001***	***15,057,000***	***81,000***	***0.151187***	***0.070502***
3	12,022,001	12,096,000	74,000	0.059019	0.037328
4	14,051,001	14,107,000	56,000	0.045428	0.017924
7	5,611,001	5,679,000	68,000	0.043934	0.022288
12	***1,246,001***	***1,324,000***	***78,000***	***0.088095***	***0.031829***
12	11,552,001	11,606,000	54,000	0.042713	0.016569
12	8,224,001	8,268,000	44,000	0.039849	0.018237
13	***996,001***	***1,079,000***	***83,000***	***0.098055***	***0.033719***
13	649,3001	6,572,000	79,000	0.057914	0.025726
13	3,695,001	3,737,000	42,000	0.039177	0.024136
14	4,805,001	4,893,000	88,000	0.04585	0.02769
21	933,001	1,010,000	77,000	0.060563	0.022807
22	14,409,001	14,478,000	69,000	0.052608	0.022232
22	14,117,001	14,165,000	48,000	0.044188	0.030042
23	183,001	335,000	152,000	0.076041	0.038974
24	3,842,001	3,921,000	79,000	0.045891	0.029727
27	8,818,001	8,865,000	47,000	0.041405	0.013411
28	10,021,001	10,087,000	66,000	0.04686	0.016208
31	4,805,001	4,858,000	53,000	0.040928	0.017554

H. zea from Vip3A-expressing plants or non-expressing refuge corn planted among Vip3A expressing plants were compared to *H. zea* collected from non-expressing pure stand refuge. Significant Weir and Cockerham's weighted F_{ST} windows were constructed from merged adjacent 40-kb windows. Mean and maximum F_{ST} calculated for merged 40 kb significant windows are presented. Italicized windows are in the top three windows by maximum F_{ST}.

frequency (19, 48). Sequencing samples from a controlled, paired plot experimental design theoretically reduces the impact of environmental variation on both phenotypes and allele frequency because all insects are collected from a similar environment (*SI Appendix*, Fig. S2). This should increase power to detect resistance-related evolutionary change. Furthermore, it can detect allele frequency differences associated with both major and minor effect resistance loci. If genomic approaches are to be used for resistance monitoring, this paired plot design could eliminate the need for follow-up functional studies, as well as complement existing damage-based sentinel Bt resistance monitoring designs that have been widely adopted by academic researchers (43, 54, 55) and have attracted interest from the US-EPA (79, 80).

Using this approach, we identified small but significant allele frequency divergence in a Cry1Ab resistance-associated genomic interval on Chr 9 within a single generation of selection, complementing previous findings from temporal sampling of wild *H. zea* (48). Widespread Cry1Ab resistance in *H. zea* represented a challenging case for detection using our genomic approach. Allele frequencies are bounded between 0 and 1, and high frequency resistance alleles present in the overall population have limited potential for increase, even under strong selection (81). Combining paired plot sampling with whole genome sequencing provides an efficient strategy for measuring selection for resistance alleles relative to simply tracking changes in a field population over time. *H. zea* uses both non-Bt crops and wild host plants as refuge, which maintains susceptible individuals in the landscape. The frequency of these susceptible individuals should vary over space and time, but likely make up ca. 10% of the overall *H. zea* population (54). When we used paired collections, toxin-expressing plants acted as filters for resistance alleles, enriching them in one sequenced population (e.g., those collected from the expressing plants) and maximizing differences between sequenced populations (e.g., the comparison between expressing and non-expressing plants). Our ability to identify resistance-related allele frequency differences in wild *H. zea* exposed to Cry1Ab toxins and pure stand refuge demonstrated the sensitivity of this experimental design for use in genomic resistance monitoring.

After documenting its sensitivity, we applied our approach to wild *H. zea* exposed to pure stand non-Bt refuge and Vip3A. Although many of our Vip3A-exposed individuals were sampled from putatively non-Bt corn in three-toxin seed-blended refuge, phenotypic data collected on these *H. zea* individuals (Fig. 1 *E* and *F*) and levels of kernel expression in refuge plants adjacent to Vip3A-expressing plants (Fig. 2) supported their exposure. Cross-pollination resulted in measurable toxin expression in refuge kernels, which decreased with refuge distance from toxin-expressing plants.

Upon hatching, the first food resource encountered by *H. zea* larvae in refuge plants is maternally derived silk tissue and the pericarp of the kernel, both of which should be non-Bt expressing. If a refuge kernel is wind-pollinated by a Vip3A-expressing plant, however, the endosperm would express Vip3A toxins, providing an opportunity for low dose exposure. This is in direct contrast to the high dose refuge resistance management approach widely adopted to slow the spread of Bt resistance. Instead of serving as a refuge for susceptible alleles, seed-blended refuge appears to provide a selective advantage to individuals capable of tolerating low doses of Vip3A toxin (49). Ultimately, low-dose exposure has potential to select for resistance at multiple variants across the genome, each with small potential to increase resistance. Resistance alleles at these variant sites accumulate in pest genomes over time, together producing toxin-tolerant phenotypes (73). In this way, seed-blended refuge may serve as a steppingstone to resistance, rather than a barrier to its evolution, allowing for accumulation of multiple resistance variants in pest genomes, and supporting the differential survival hypothesis posited by Onstad et al. (82).

Recent studies have described unexpected infestation and injury in Vip3A expressing corn (52, 55), yet clearly attributing this

injury to heritable Vip3A resistance has been a challenge. Follow-up studies of populations collected from plots with unexpected Vip3A infestations may continue to indicate efficacy if the scoreable phenotype is mortality on transgenic Vip3A expressing ears (55). In our work, populations collected from Vip3A contaminated refuge may have died if placed directly onto Vip3A expressing ears, because they likely do not yet have a mutation with a large enough phenotypic effect to tolerate a full dose of Vip3A. Yet it is well known that low doses of insecticidal compounds are more likely to select for polygenic resistance (73). In the case of *H. zea*, exposure to three-toxin seed-blended refuge may provide the opportunity for resistance mutations of moderate and small effect size to accumulate over time, allowing for tolerance of full strength Vip3A toxin. We have already seen this in the case of *H. zea* tolerance to Cry toxins (48). Our work, which aims to document emerging resistance, indicates that *H. zea* may already be on a similar evolutionary trajectory for Vip3A.

Upon sequencing Vip3A-exposed individuals, we identified several regions of the genome, whose patterns of genomic change were consistent with multi-genic Vip3A-associated selection upon standing genetic variation, as would be expected under the evolutionary conditions described above. One particularly compelling region of genomic divergence between Vip3A-exposed individuals and those from pure stand non-Bt refuge was found on Chr13, within a few Kb of a tenascin-like gene thought to bind Vip3A toxins (74). While our results support selection for Vip3A tolerance at this genomic region, further functional work will be required to fully elucidate the contributions of tenascin-like, and other genes to emerging Vip3A tolerance wild *H. zea* (9).

We have applied genomic monitoring to track Bt resistance-associated allele frequencies in the overall *H. zea* population over time (48) and, in the present study, to calculate differences between paired plot collections in a single generation. Yet tracking allele frequency differences over time in paired plot collections at multiple locations would present the most powerful evidence of resistance emergence. Sustained allele frequency divergence between toxin-exposed and unexposed individuals across multiple years would provide evidence for selection, as opposed to allele frequency fluctuations caused by annual migration-related founder effects, seasonal population demographic shifts, or changes in population size. Future empirical work should focus on examination of resistance-related temporal genomic divergence in paired plot collections to further refine our understanding of the utility of genomic monitoring. Moreover, while genomic monitoring approaches have been empirically tested for Bt resistance monitoring, where the pesticide is expressed by a transgenic plant throughout the growing season, there is potential for its adaptation to sprayable technologies for management of other insect and weed pests, or fungal pathogens. Careful consideration of the timing and frequency of sampling for pesticide exposed and unexposed individuals would be necessary for extension to resistance management programs that do not involve plant-incorporated pesticidal technologies.

Methods

Paired Plot Experimental Design and *H. zea* Field Collection. Experimental plots of Bt expressing field corn and their non-Bt expressing near isoline were planted in 2019 and 2020, at the North Carolina State University Vernon James Research and Extension Center in Plymouth, NC. These plots were part of a larger multi-state survey of the lethal and sublethal impacts of Bt corn on *H. zea* published by Pezzini et al. (49). Seeds of DuPont Pioneer 1637R (non-Bt expressing), 1637YHR (Cry1Ab+Cry1F) and 1637VYHR (Cry1Ab+Cry1F+Vip3Aa20) were planted as pure stands and in 80:20 blends of Bt:non-Bt seed resulting in five replicated plot types (Fig. 1 *A*–*C*) and seven treatment groups: 1) pure stand non-Bt field corn, 2) pure stand two-toxin expressing corn, 3) two-toxin expressing corn in seed-blend refuge plots, 4) non-Bt refuge plants among two-toxin expressing corn, 5) pure stand three-toxin expressing corn, 6) three-toxin expressing corn in seed-blend refuge plots, and 7) non-Bt refuge plants among three-toxin expressing corn. *H. zea* pre-pupae emerging from corn were allowed to pupate in soil. Further details on plot design can be found in *SI Appendix, Supplemental Methods*.

Analysis of Infestation Rate and Pupal Weight from NC Field Collections. Pupae were excavated from the soil, and pupal weight, survival, and sex were recorded. Pupae were reared to adulthood, and newly emerged moths were frozen until genomic analysis. Generalized linear models tested the effect of treatment on the number of pupae collected per 20 ears and pupal weight [(83); v.3.4.4]. Tukey–Kramer adjusted pairwise comparisons of least square means were used to determine significant differences ($\alpha = 0.05$) among treatments [multcomp, *glht*; (84)].

Vip3A Expression Tests. Vip3A toxin expression was quantified in ears grown from separated expressing and non-expressing corn seeds of Trecepta 40-99-RIB (Trecepta DKC40-99™, Bayer Crop Science). Four rows of Vip3Aa20 expressing field corn were planted next to four rows of non-expressing corn (*SI Appendix, Supplemental Methods*). Upon maturity, 10 ears were harvested from the furthest and closest rows of each variety for a total of 40 ears across four conditions. Twenty kernels were removed from each replicate ear and ground into a fine powder using a coffee grinder. Approximately 125 mg of pooled kernel tissue per ear per condition was tested with QuickStix (EnviroLogix) for Vip3A expression according to manufacturer instructions. As a second semi-quantitative measure of expression, brightness of the test band relative to the control band was calculated for each sample using digital images of the QuickStix test strips in ImageJ (85). ANOVA followed by post-hoc t-tests with Bonferroni corrections were used to identify statistically significant differences in band brightness between conditions [(86), v. 4.1.1]. QuickStix expression test results were validated by qPCR for a subset of samples using methods modified from Liang et al. [(58); *SI Appendix, Supplemental Methods*].

Sequencing, Alignment, and Genotype Filtering. DNA from 154 *H. zea* samples collected in NC were prepared into Illumina TruSeq libraries and sequenced on two NovaSeq 6000 lanes with 150-bp paired end sequencing. Most samples were from 2019 collections, but 3 from Vip3A exposure treatments were from 2020 to increase our statistical power to detect signatures of Vip3A selection. After filter-trimming, Bowtie [(87), v. 2.2.5] read alignment to a chromosome scale *H. zea* assembly [(88), v. 1.0, PRJNA767434], variant calling with bcftools [(89), v. 1.11] and mpileup, 151 samples were used for downstream population genomic analysis.

Analysis of Population Genomic Structure and Window-Specific Divergence. To characterize major patterns of genomic variation in *H. zea* collected from NC treatment plots, a principal component analysis was performed on genome wide SNP data using Plink [(90), v. 1.90]. We then compared individuals from Cry1Ab+Cry1F expressing structured plots and seed-blend plots (including conventional refuge planted among Cry1Ab+Cry1F expressing plants that likely had been cross-pollinated) to individuals collected from pure stand refuge. We also compared individuals from the seed-blend plots expressing Cry1Ab+Cry1F+Vip3Aa to individuals collected from pure stand refuge. Genome-wide divergence and window-specific divergence for these treatment pairs were calculated using Weir and Cockerham's weighted F_{ST} (59) for 10-, 20-, and 40-kb windows with 1-kb steps in VCFtools. Analysis of multiple window/step sizes is reported in *SI Appendix, Supplemental Results*, but only results from the 40-kb windows are reported in the main text. Overall pairwise genome-wide divergence was first calculated as the average weighted F_{ST} calculated from 40-kb sliding windows for all genomic windows excluding those with <10 SNPs. To identify specific windows putatively under selection for each pairwise comparison, we calculated z-transformed F_{ST} values. Windows with zF_{ST} values greater than 6 were considered to have undergone statistically significant divergence (91) indicative of selection. Continuous (overlapping or directly adjacent) 40-kb significant F_{ST} windows were merged to form candidate windows of various sizes for each pairwise comparison.

We investigated the potential for unequal sample sizes or sex ratio bias to influence our F_{ST} estimates (*SI Appendix, Supplemental Methods, Supplemental Results*, and Figs. S10 and S11). For the most unequal group size comparison we conducted, we estimated F_{ST} for 1,000 equal sample size bootstrap subsets of individuals. The genome-wide significance threshold and F_{ST} was compared between the full dataset and the 1,000 bootstrap replicates. To mitigate potential impacts of sex ratio bias and the different effective population sizes for the sex chromosomes and autosomes, zF_{ST} significance thresholds were set separately for the Z chromosome (Chr 1) and the autosomes (60).

We further characterized signals of Vip3A-related selection in the genomic windows with the strongest signals of toxin-related divergence. We visualized signals of linkage disequilibrium with r^2 heatmaps made with LDBlockShow [(92), v. 1.4]. Signals of genetic distance between individuals exposed to Vip3A toxins and those from structured non-Bt refuge were explored at these genomic regions by calculating pairwise genetic distance between individuals to construct a neighbor-joining phylogenetic tree with the R package ape [(93), v. 5.7]

Candidate Gene Identification. Gene information was lifted from the ilHelZeax1.1 genome assembly (GCA_022581195.1) and Hzea_1.0 [PRJNA378438; (94)] (as ilHelZeax1.1 was released after these analyses were begun using the *H. zea* GA-R chromosome scale assembly (GCA_022343045.1)) using the program liftoff with default parameters (95). Any genes fully or partially overlapping with merged significant windows were considered as potential candidates and functional information was extracted from both lifted-over annotations. Cry and Vip3A resistance candidate genes were identified from previous studies (reviewed in refs. 96–99). Additionally, the gene sequence for Vip3A toxin–related candidate genes (75–78, 100) was downloaded from genbank and compared to the *H. zea* GA-R chromosome scale assembly to confirm position and identity using local Blast [(101), v. 2.9.0].

Data, Materials, and Software Availability. Whole genome sequencing data from North Carolina populations of *H. zea* have been deposited at NCBI under BioProject ID PRJNA1055981 (102). Larval midgut RNA sequencing reads have been deposited at NCBI under BioProject ID PRJNA1055985 (103). Scripts for whole genome and transcriptomic analyses can be found at https://github.com/TaylorKT/zea_genomic_monitoring (104). Data and scripts for qPCR analysis of Vip3A expression can be found at https://github.com/mcadamme/CornExp_Vip3A (105).

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