# Polygenic basis of field evolved Bt Cry resistance in the corn earworm, *Helicoverpa zea*

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**Abstract**

In agricultural ecosystems, strong and shifting selective pressures from pest management practices can lead to rapid evolution. Crops expressing pesticidal proteins from *Bacillus thuringiensis* (Bt) are an important control measure for agricultural pests and a major selective force on pest genomes world wide. *Helicoverpa zea*, a destructive pest of corn and cotton, has evolved widespread resistance to Bt crystalline (Cry) pesticidal proteins. Here we reveal the polygenic architecture of field evolved Cry resistance in *H. zea* and identify multiple previously unknown Cry resistance candidate regions and genes. In the genomic region with the largest effect on Cry1Ab, and Cry1A.105 + Cry2Ab2 resistance, we identified signals of an increase in copy number for an entire cluster of trypsin genes. The genes in this cluster, along with other trypsins genome-wide, are significantly upregulated in Cry resistant *H. zea*. All field resistant individuals possessed two to four copies of a trypsin in this cluster, but this gene amplification was not found in any samples collected prior to Cry resistance evolution or in susceptible lab colonies. Inhibiting trypsin activity in *H. zea* decreased tolerance of Cry toxin in resistant and susceptible populations. Together, our findings suggest that increased expression and amplification of trypsins could allow degradation of activated Cry toxin and explain part of the polygenic Cry resistance observed in wild *H. zea.*

## Introduction

Human activity has modified ecosystems worldwide from urban landscapes to agricultural fields (Lewis & Maslin, 2015). Anthropogenic changes to the landscape impose strong and shifting selection pressures that broadly impact ecosystems and shape the phenomes and genomes of the organisms contained within them (Palumbi, 2001). The responses to selection in affected species may have significant negative impacts on human lives, such as in cases of antibiotic and pesticide resistance evolution (Fair & Tor, 2014; Gould et al., 2018). Understanding how human mediated selective pressures rapidly change the evolutionary trajectories of other organisms will be key to predicting and mitigating potential impacts on ecosystems and human lives.

Cultural and chemical management practices used in agroecosystems exert selective pressure on pests to evade management through resistance evolution. Strong resistance to these practices can evolve rapidly. One meta-analysis estimated that resistance evolved on average within 14 years of the release of a new chemical control measure (Brevik et al., 2018). In arthropods alone, resistance to over 300 pesticidal compounds in more than 600 species has been documented (Fritz, 2022; Mota-Sanchez & Wise, 2022). Keeping pace with pest resistance evolution is a significant challenge, as discovery and development of new control measures is time consuming and costly (Gould et al., 2018; Sparks, 2013). Despite control efforts, weeds, disease, and animal pests together result in estimated yield losses of around 20 - 30% in major food crops (Oerke, 2006; Popp et al., 2013; Savary et al., 2019). Pest resistance evolution causes billions of dollars in crop losses every year, and further pest management failures will lead to additional crop loss and significant economic impacts (Gould et al., 2018; Palumbi, 2001). Understanding and tracking how pest populations are responding to control measures will be one important component of maintaining sustainable and productive agricultural systems.

In 1996, deployment of genetically engineered crops producing insecticidal proteins from *Bacillus thuringensis* (Bt) was a major innovation in lepidopteran and coleopteran pest management worldwide. The pesticidal proteins expressed in Bt crops are highly targeted insecticides, making them safe for ecosystems and human health (Koch et al., 2015; Mendelsohn et al., 2003; Sanahuja et al., 2011). Since their introduction, Bt crops have suppressed pests area-wide leading to increased yield and profits while decreasing reliance on harmful broad-spectrum insecticides (Cattaneo et al., 2006; Dively et al., 2018; Hutchison et al., 2010; Kathage & Qaim, 2012; Perry et al., 2016). In 2023, 85% of corn and 89% of cotton planted in the United States expressed Bt traits (USDA-ERS, 2023). Such widespread use makes Bt crops a major selective force in agroecosystems (Gassmann & Reisig, 2023; Tabashnik & Carrière, 2017). Despite mitigation efforts, practical resistance to Bt crops has evolved in several major pest species, including *Helicoverpa zea* (Tabashnik & Carrière, 2017).

The corn earworm, *H. zea*, is a polyphagous lepidopteran pest which causes significant damage to corn and cotton. Bt crops expressing crystalline (Cry) proteins successfully managed *H. zea* when they first became available in 1996. In the years following release, susceptibility to Cry proteins in *H. zea* decreased and crop damage increased (Dively et al., 2016). Although multi-toxin cultivars expressing novel Cry toxins were developed and released, widespread and damaging resistance to these cultivars was documented in *H. zea* by 2016 (Dively et al., 2016; Yang et al., 2022). To slow resistance evolution, Bt crops are ideally designed to produce a "high" dose of toxin, or 25 times the amount of toxin needed to cause mortality in susceptible insects (US EPA-SRP, 1998). Non-expressing plants, or "refuge", are planted near expressing plants, and produce homozygous susceptible pest individuals that swamp out resistance alleles present in the local landscape (Gould, 1998; Huang et al., 2011). Resistance evolution in *H. zea*

was predicted because Cry expressing Bt crops do not produce a “high” dose of toxin for *H. zea* (Horner et al., 2003), and refuge implementation for corn and cotton is often insufficient (Reisig, 2017; Reisig & Kurtz, 2018). Resistance may also be facilitated by cross pollination between expressing and refuge corn, which produces kernels expressing low Bt toxin doses (Pezzini and Taylor et al., 2024; Dively et al., 2020; Yang et al., 2015). *H. zea* is now primarily controlled by Bt crops expressing a pyramid of Cry and vegetative insecticidal proteins (Vip) toxins, but early warning signs of Vip resistance evolution in *H. zea* are already being detected (Yang et al., 2019, 2020, 2021). A better understanding of the mechanisms and evolution of Cry resistance in *H. zea* might allow the preservation of remaining Cry and Vip efficacy, or development and deployment of more durable control measures in the future.

Cry protein toxicity is generally understood to begin with the Cry protoxin being solubilized and activated by proteolytic enzymes in the insect digestive system. However, this activation step is not necessary for activated toxins expressed throughout the tissues of many Bt crops (Clark et al., 2005; Gould, 1998). Activated toxins then interact with a series of midgut receptors, eventually causing lysis of the midgut epithelial cells and leading to growth suppression or mortality in targeted insects (Heckel, 2020; Jurat-Fuentes et al., 2021; L. Liu et al., 2021). The multi-step and complex molecular mechanisms of Cry toxicity underlie a wide variety of potential mechanisms of resistance to Bt crops. Bt resistance in other species has generally been connected to the disruption of the midgut toxin binding sites or reduced expression of target genes, although there are also examples of resistance associated with altered Bt protein processing, detoxification, and changes in immune function (Heckel, 2020; Jurat-Fuentes et al., 2021; L. Liu et al., 2021).

We recently demonstrated that field evolved Cry1Ab and Cry1A.105 + Cry2Ab2 resistance in *H. zea* was polygenic, but the full genetic architecture remained unknown (Taylor et al., 2021). Genome scanning of field-collected *H. zea* collected in the years following commercialization of Bt crops led us to reject most of the common Cry resistance mechanisms described in other species, and instead pointed to novel resistance-related genomic regions (Taylor et al., 2021). Here, we narrowed in on these genomic regions involved in field evolved Cry1Ab and Cry1A.105 + Cry2Ab2 resistance, described variation in resistant genotypes among field-collected *H. zea* populations, and identified genome-wide resistance-related changes in gene expression. Although multiple novel regions and candidate genes appear to underlie Cry resistance in *H. zea*, we identified a ~150 kb genomic region of major effect on resistance, which contained a cluster of 7 trypsin genes. This gene cluster appeared duplicated or quadrupled in all resistant field-collected *H. zea,* yet susceptible lab colonies and field-collected samples pre-dating resistance evolution contained only a single copy. We linked this gene amplification to an increase in expression of all 7 trypsins in resistant individuals. We also found that several other trypsins outside of this cluster were among the most differentially expressed genes genome-wide in resistant individuals. We showed that inhibiting trypsin activity significantly reduced Cry tolerance in resistant and susceptible caterpillars in laboratory assays. These results led us to propose that gene amplification and increased expression of trypsins could lead to rapid Cry toxin degradation and resistance in *H. zea.*

## Results

### Cry resistance phenotypes

We measured Cry resistance and growth phenotypes in field resistant and laboratory susceptible populations, and in $F_2$ hybrid offspring from 10 intercross families. Phenotypes were

measured in laboratory growth assays with diets containing either a diagnostic dose of Cry1Ab or Cry1A.105 + Cry2Ab2 expressing corn leaf tissue or leaf tissue from the non-expressing corn near isolines as described in Dively et al. (2016) and Taylor et al. (2021). Briefly, weight gain was measured after seven days of feeding on the aforementioned diets and comparisons between them enabled us to discriminate Cry resistance from general survival and growth advantages and identify any fitness effect of resistance alleles (**Figure 1, Figure S1, Table S1, Table S2)**. The $F_2$ offspring phenotypes suggested that resistance to all the Cry toxins we tested and growth in the control treatments were quantitative genetic traits (**SI Results**).

**Sequencing and variant calling**

Parents and progeny from 10 replicated intercross families were sequenced on an Illumina NovaSeq 6000. Short reads were trimmed and filtered for quality and aligned to a *H. zea* chromosome scale assembly (v. 1.0, PRJNA767434; Benowitz et al., 2022). Whole genome sequencing of the 20 cross parents resulted in a total of 4,716,009 high quality genome wide single nucleotide polymorphisms (SNPs) identified in the resistant and susceptible populations. The genome wide average sequencing coverage of 18.6× (*st. dev.* = 2.4) ensured accuracy of the genotyping calls. Divergence between the field collected resistant and laboratory susceptible founding populations was high genome wide (**Figure S3**, **Figure S4, SI Results**), which highlighted the importance of laboratory cross experiments for the detection of genomic regions involved in resistance.

The sequencing of $F_2$ offspring at double digest restriction site associated DNA (ddRAD) sequencing markers resulted in 78,580 - 79,408 high quality SNP markers for genotype-phenotype association in each treatment. Final SNP marker numbers likely varied because of true difference in SNP presence, different loci passing quality control filters, and

genetic variation among individuals at restriction cut sites used for marker development (Davey et al., 2013). The average sequencing coverage of a ddRAD locus across individuals was 52,225× (*st. dev.* 45,011) suggesting high but variable coverage of loci and an average coverage for a single individual of 63× . For identification of the direction of allele effect, the ddRAD SNP sets were further filtered to include only those variants where the allele origin population (field resistant vs. lab susceptible) could be reliably predicted. This produced the smaller filtered sets of 6,717 - 6,749 genome wide SNPs for each treatment that were used for visualization of effect size and direction.

**Cry resistance trait architecture**

Cry resistance trait genetic architecture was described by hyperparameter estimates from bayesian sparse linear mixed models (BSLMM) in gemma (Zhou et al., 2013). The proportion of phenotypic variance explained by the full panel of genetic markers sequenced for the $F_2$ offspring (PVE) was high for both Cry resistance traits with mean estimates of 69.7% [95% CI = 45.4 - 93.7] for the Cry1Ab treatment and 71.1% [95% CI = 43.6 - 96.5] for the Cry1A.105 + Cry2Ab2 toxin blend, and lower for the control growth treatments 44.7% [95% CI = 20.3 - 71.9] and 51.2% [95% CI = 27.1 - 74.1] (**Figure S2**). The number of variants of large effect detected were similar across treatments with mean estimates ranging from 11.3 - 34.1. In all cases, there were wide overlapping credible intervals around these means, suggesting that tens of genomic variants of major effect underlie phenotypic differences in these traits (**Figure S2**). These variants of large effect underlie 74.0% [95% CI = 36.9 - 98.6] of the phenotypic variance explained by the full panel of genomic variants (PGE) for the Cry1Ab treatment, 77.8% [95% CI = 46.8 - 98.9] for the Cry1A.105 + Cry2Ab2 treatment and 53.4% [95% CI = 0.5 - 97.3%] and 73.6% [95% CI = 24 - 99.2] for the control treatments (**Figure S2**). These results further support a polygenic

genomic architecture of Cry resistance, as suggested by the phenotypic data. Notably, the high estimates for PVE and PGE for both Cry toxin treatments suggest that our data set and analysis were able to identify most of the genetic basis of field evolved Cry resistance phenotype in *H. zea*.

**Genomic regions underlying Bt Cry resistance**

The genomic regions underlying field evolved Cry resistance were detected by the association of genome wide ddRAD markers with weight gain after seven days of feeding in Cry toxin or control leaf incorporation assays. Genotype-phenotype association was measured in $F_2$ intercross offspring of field-collected resistant and laboratory susceptible *H. zea*. Using a linear mixed model (LMM), we estimated the additive effect (β) of a single resistant population allele on weight gain in mg. Smoothed additive effects of genetic markers across the genome on growth phenotypes are shown for Cry1Ab, Cry1A.105 + Cry2Ab2, and control treatments (**Figure 2**). The quantitative trait loci (QTL) shown in Figure 2 were also identified with multiple SNP filtering criteria and in the unsmoothed data set (**Figure S5** and **S6)**.

We detected that a resistant parent allele in $F_2$ offspring significantly increased growth in the Cry1Ab treatment in regions of Chromosomes (Chr) 2, 3, 6, 9, and 30 with a significance threshold of Bonferroni corrected $p < 0.01$. With the less stringent criteria of Bonferroni corrected $p < 0.05$ we also detected an association of parts of Chr 11 and 21 (**Figure 2**). For the Cry1A.105 + Cry2Ab2 containing diet we only detected a significant effect of Chr 9, though we also saw non-significant patterns suggesting possible shared QTL with the Cry1Ab treatment on Chr 2 and 30. We detected genomic regions associated with general growth in the laboratory assays on Chr 10, 14 and 27 (**Figure 2**). The effect of Chr 10 on growth is also detected in the Cry1Ab treatment, but we do not consider this region to be Cry resistance related because the

220 association with growth was found in both toxin containing and control treatments. The negative
221 direction of this effect suggests that the growth associated allele is more common in the
222 susceptible parent population. Importantly, we did not detect any fitness cost in non-Cry
223 expressing treatments for any of the Cry resistance associated genetic variants (**Figure 2**).

224 On both Cry treatments, we found that the region of the genome with the largest additive
225 effect on resistance was on Chr 9. In the Cry1Ab treatment we identified a clear peak between 5
226 and 6 Mb on Chr 9. We previously identified signals of divergence between field collected
227 samples from 2002 and 2017 at 5.75 Mb on Chr 9 (Taylor et al 2021). In this analysis we also
228 identified a directly overlapping divergence peak at 5.75 Mb between the resistant and
229 susceptible cross parents (SI Results). The region between 5 and 6 Mb contained seven trypsins
230 and several cytochrome b5-related proteins (**Table S3**) and was examined further below. The
231 QTL on Chr 30 had a clear peak at ~2.5 Mb, making it possible to describe candidates near that
232 peak. Within 100 Kb of the Chr 30 peak (between 2.4 - 2.6 Mb) were a MAP kinase-activated
233 protein kinase 2-like, HzeaTryp129, a cytochrome P450, and HzeaABCC11, toll-like receptors,
234 carboxylesterases, and carboxylase-like genes suggesting that several known Bt candidate gene
235 families and insecticide resistance related genes could underlie this QTL. The other identified
236 QTL on Chromosomes 2, 3 and 6 had more diffuse genomic signals with less well-defined
237 peaks, making it difficult to identify narrow regions most linked to resistance. In the SI Results,
238 we further described limited evidence for any role of other major candidate genes.

239 **Resistance associated differential gene expression**

240 We identified differentially expressed genes in the larval midgut between field resistant
241 and laboratory susceptible populations as well as potential Cry inducible expression changes
242 between field populations collected from Cry expressing and non-expressing corn. In total, we

243 analyzed 14,600 annotated genes genome-wide. In the field resistant populations, there were 527
244 genes significantly upregulated and 352 genes significantly downregulated relative to the
245 susceptible population (p-adjusted < 0.01; **Table S4**). Several candidate gene families with roles
246 in Bt resistance have members among the most differentially expressed genes between resistant
247 and susceptible populations. Twelve of the top 50 most differentially expressed genes were
248 upregulated trypsins (**Table S4**). Seven significantly differentially expressed trypsins were found
249 between 5 and 6 Mb on Chr 9, and six of those are among the most differentially expressed genes
250 genome wide. Additionally, among the top 50 differentially expressed genes, we see a
251 downregulated cadherin-87A-like gene and a downregulated cytochrome P450 (CYP301B1) in
252 resistant individuals. Although not among the top differentially expressed genes, another
253 cytochrome P450 (CYP6AB38) is significantly upregulated in resistant individuals and found
254 close to strong signals of genotype and resistance phenotype association on Chr 2.

255 Between resistant populations collected from Cry expressing and non-expressing corn,
256 there were 13 genes significantly upregulated and 20 genes significantly downregulated in
257 caterpillars collected from expressing corn (**Table S5**). Four trypsins and one chymotrypsin were
258 among the most upregulated genes in Cry exposed resistant individuals. Only one of the trypsins
259 found between 5 and 6 Mb on Chr 9, Trypsin 80, was in this group and showed signs of Cry
260 inducible changes in expression. Downregulated genes in Cry exposed individuals included a
261 cadherin-87A-like gene and Lysozyme1, an immune related gene.

262 **Potential genetic mechanism of large effect on Bt Cry resistance**

263 Taken together, evidence from the QTL and differential expression analysis suggested
264 that the trypsin cluster on Chromosome 9 likely plays an important role in Bt Cry resistance.
265 Closer investigation of this region revealed signals of a potential structural variant. From 5.22 -

266 5.37 Mb on Chromosome 9, a region encompassing the full cluster of seven trypsins from 5.27 -
267 5.34 Mb, resistant field samples had increased whole genome sequencing (WGS) coverage depth
268 relative to each individual's genome wide average sequencing depth **(Figure 3)**. This signal was
269 not found in WGS data from the susceptible lab population or in field samples collected in 2002
270 before the evolution of Cry resistance. In WGS data from individuals collected in the field after
271 the documentation of widespread Cry resistance, in 2017 and 2019, we detected signals of the
272 structural variant in most samples. We detected stronger coverage signals in individuals from
273 2019 compared to 2017, except perhaps in some individuals from non-Bt expressing corn
274 **(Figure 4)**. These coverage signals suggested a duplication of this region in the genomes of
275 resistant individuals. The increased depth of coverage detected between 2017 and 2019 could
276 reflect increased numbers of individuals homozygous for this variant as it increased in frequency
277 in the population. The absence of this variant in 2002 samples suggests that it may have occurred
278 first after that time or was a local or rare allele not sampled.

279 The observed upregulation of all trypsins in this cluster in resistant individuals was
280 consistent with duplication of these genes **(Figure 3)**. Using droplet digital polymerase chain
281 reaction (ddPCR), we quantified the copy number variation for one gene in the trypsin cluster
282 (Trypsin 77) to confirm that the coverage and differential expression signals we detected were
283 truly related to gene amplification **(Figure 4)**. Our copy number variant analysis showed that all
284 resistant individuals had 2 to 4 copies of Trypsin 77, while all susceptible individuals had only
285 one. The variance in copy number observed in resistant individuals may represent heterozygosity
286 or different numbers of duplications. In either case, it would suggest that this resistance genotype
287 is not fixed in the resistant field population. We also showed a strong relationship between
288 detected copy number and relative coverage depth of the trypsin cluster ($r^2 = 0.95$), suggesting

289 that coverage depth is a reasonable proxy measure for copy number in this case **(Figure 4)**. We
290 demonstrated that ddRADSeq coverage of this trypsin cluster was also significantly associated
291 with growth phenotypes (**Figure S7**, **SI Results**). Notably, we also found that the $F_2$ offspring of
292 the family that grew the least on the Cry1Ab treatment had no variation in coverage in this
293 region and had a small estimated effect size of all SNPs on Chr 9 (**Figure 4**). These results
294 together suggest that the gene amplification or another very tightly linked variant explains most
295 or all of the association between Chr 9 and Cry resistance phenotypes.

296 **Synergistic effect of trypsin inhibition and a Cry toxin**

297 To functionally link trypsin activity from the Chr 9 gene amplification and the other
298 upregulated trypsins to Cry tolerance in *H. zea*, we tested if inhibiting trypsins with
299 N-α-tosyl-ι-lysine chloromethyl ketone (TLCK) reduced tolerance to Cry proteins in resistant
300 and susceptible populations. The buffer alone did not impact growth ($p > 0.05$, data not shown),
301 therefore we used the buffer control treatment for all further analyses and visualization. We
302 compared growth on laboratory diets with increasing TLCK concentrations and a diagnostic dose
303 of Cry1Ab expressing corn leaf tissue or leaf tissue from the non-expressing near isoline. To
304 separate the effects of Cry toxin and TLCK alone from any combined effect, we used a model
305 reduction and comparison approach, and identified a significant interaction between trypsin
306 inhibition with TLCK and Cry1Ab presence for both resistant and susceptible *H. zea* populations
307 ($p < 0.001$) (**Figure 5, Table S6**). Post hoc contrasts of the estimated marginal means confirmed
308 that a trypsin inhibitor paired with a Cry toxin significantly decreased larval growth (**Table S7**).
309 We also calculated a growth ratio, which compared growth on Cry expressing and non
310 expressing treatment at each TLCK dose. If trypsins were not strongly involved in Cry
311 resistance, we reasoned that the ratio in growth suppression should remain constant with

312 increasing doses of a trypsin inhibitor. Instead this growth ratio decreased as TLCK dose
313 increased, a pattern suggesting that Cry toxicity and trypsin activity are related **(Figure 5)**. In the
314 Cry1Ab and 10X TLCK treatment group weight in the resistant population was 17.4 mg on
315 average, suggesting growth suppression similar to the susceptible population on Cry toxins
316 alone, which averaged 20.7 mg (**Table S6)**. These results indicate that trypsin activity is a
317 protective factor for both resistant and susceptible *H. zea* in the presence of leaf material
318 expressing Cry1Ab toxin.

319 **Discussion**

320 Here we identified multiple genes and genomic regions linked to field-evolved Cry
321 resistance in *H. zea*. Using QTL analysis, we identified multiple genomic regions associated with
322 Bt resistance, which together explain the majority of phenotypic variance in Cry resistance
323 observed across 10 replicated $F_2$ mapping families. Our findings support a polygenic basis of Cry
324 resistance in *H. zea*, but the genomic region of largest effect on both Cry1Ab and Cry1A.105 +
325 Cry2Ab2 resistance is found on Chromosome 9 (**Figure 2**). We identified a narrow
326 genotype-phenotype association peak between 5 and 6 Mb on Chromosome 9 that encompasses a
327 cluster of 7 trypsin genes (**Figure 3**), a gene family previously thought to be involved in
328 lepidopteran Cry resistance via toxin activation or degradation (Gong et al., 2020;
329 González-Cabrera et al., 2013; C. Liu et al., 2014; Ma et al., 2013). A region in this genomic
330 window changed significantly as Cry resistance evolved between 2002 and 2017 and continued
331 to diverge in Cry tolerant *H. zea* relative to the general population in 2019 and 2020 (Pezzini and
332 Taylor et al. 2024; Taylor et al., 2021). We found that multiple trypsin genes in this region, along
333 with several other trypsins genome-wide, are among the most upregulated genes in resistant *H.*
334 *zea* **(Figure 3, Table S4)**.

335 We found that the region between 5.22 - 5.37 Mb on *H. zea* Chr 9, containing seven
336 significantly upregulated trypsins and the QTL mapping peak, showed strong evidence of at least
337 one duplication in all of the resistant cross founding parents. This gene amplification was
338 confirmed with copy number variant analysis by ddPCR. Signals of the trypsin gene
339 amplification are absent in all samples tested from the susceptible lab colony and field samples
340 from 2002 that pre-date Cry resistance evolution. The lack of detection in 2002 suggests that
341 selection for resistance may have acted on a novel or rare genetic variant. The signals of this
342 gene amplification increased in the population between 2017 and 2019, suggesting that in those
343 years the duplication became more common or potentially additional duplications occurred,
344 leading to field resistant individuals with four copies of these trypsins. It was not possible to
345 reconstruct the full sequence of this large structural variant with the short read sequencing
346 approaches used in this study. Future studies could employ long read sequencing to target this
347 and other resistance related structural variants.

348 Our findings suggest that wild resistant *H. zea* collected from Maryland in 2019 and 2020
349 possess two or four copies of a trypsin in the cluster, and that while both variants are associated
350 with resistance, four copies may yield stronger resistance phenotypes than two copies (**Figure 4**
351 **I&J**). All Chr 9 SNP exhibited low estimated effect size for the single mapping family that
352 lacked trypsin copy number variation. Together with the apparent relationship between copy
353 number variation and mapping family growth, this suggests that this gene amplification likely
354 explains much if not all of the detected effect of Chr 9 alleles on Cry resistance (**Figure S7,**
355 **Figure 4I&J**). However, we cannot rule out the possibility that another genetic variant tightly
356 linked to this gene amplification might be what truly underlies resistance, nor can we identify
357 with certainty how this large duplication might give rise to resistance.

358 Our findings along with what we know about mechanisms of Cry resistance in other
359 lepidoptera suggest that a likely resistance mechanism is that this gene amplification causes
360 increased expression of these trypsins which, along with upregulation of trypsins in other
361 genomic regions, degrade activated Cry1 toxins. Some of those trypsins showed signs of Cry
362 inducible up regulation (**Table S5**), but most of the trypsins in the Chr 9 cluster did not. Gene
363 amplification could explain some or all of the apparent upregulation of the trypsin cluster on Chr
364 9, as duplications often yield expression increases and in some cases those increases may be
365 greater than 2-fold (Heckel, 2022; Loehlin et al., 2022). Future work could separate the specific
366 roles of duplication and upregulation in the expression of this gene cluster.

367 Together these results suggest that trypsin activity and thus likely proteolysis play an
368 important role in field evolved Bt Cry resistance in *H. zea*. Previous work has also linked trypsin
369 activity to Cry resistance in *H. zea*, for example, Lawrie et al. (2020) found strong signals of
370 trypsin upregulation in resistant *H. zea* while Zhang et al. (2019) found that trypsin down
371 regulation was related to *H. zea* Cry resistance in a laboratory selected line. The upregulation of
372 trypsins in resistant individuals observed here suggests degradation rather than activation as the
373 potential mechanism for these genes' involvement in resistance. This would be expected in the
374 case of resistance to crops that express activated toxin. Phenotypic resistance due to Cry toxin
375 degradation by trypsins has been reported in field populations of *Spodoptera exigua* (Ma et al.,
376 2013).

377 We began to functionally link trypsin activity to Cry tolerance in *H. zea* and demonstrated
378 that trypsin inhibition synergistically reduced growth in Cry containing treatments for both
379 resistant and susceptible populations. These findings demonstrated a significant protective effect
380 of trypsin activity in Cry exposed *H. zea*. When exposed to the highest TLCK dose and Cry toxin

381 together, growth in the resistant population was suppressed to the level of the susceptible
382 population on Cry toxin alone. The shared response of susceptible and resistant populations to
383 TLCK in this assay suggests that the increased expression and duplication of trypsins might
384 allow the resistant population to better utilize a mechanism already present in the susceptible
385 population.

386 Trypsins, and specifically the gene amplification on Chr 9, likely play an important role
387 in resistance, but they are not the only candidates. We also identified resistance associated
388 genomic regions on Chr 2, 3, 6, and 30. Multiple promising candidate genes were found in the
389 QTL peak on Chr 30 and a significantly differentially expressed cytochrome p450 was identified
390 in another QTL region. There were also multiple candidate Bt resistance genes among the top
391 differentially expressed genes between resistant and susceptible *H. zea,* including a cadherin
392 gene. The genomic architecture of Bt resistance likely involves some of these candidates of large
393 to moderate effect size, which act together with other candidates we have not yet identified to
394 produce polygenic Cry resistance in *H. zea*. Further functional validation will be necessary to
395 identify specific genes underlying resistance, including experiments to knockout individual
396 candidate genes to confirm (or refute) association with resistance, as has been done in *H. zea*
397 previously (Fabrick et al., 2022; Perera et al., 2021).

398 In our non-expressing control growth treatments, we did not detect any fitness costs of
399 this Cry resistance associated gene amplification, or any of the other detected major effect loci
400 (**Figure 2**, **Figure S7**). The lack of fitness cost for resistance, the low dose of the toxins,
401 incomplete refuge implementation, and cross pollination, likely contributed together to the
402 failure of Cry resistance management strategies for *H. zea*. A degradation mechanism for toxin
403 resistance could explain the rapid evolution of resistance to multiple toxins that do not share

binding sites. Our findings link the same gene amplification to resistance to both Cry1Ab and a blend of Cry1A.105 + Cry2Ab2. It may also be associated with resistance to other Cry toxins not tested here. If, as our results suggest, degradation of activated toxin by trypsins truly underlies Bt resistance phenotypes in field *H. zea* populations, then targeted synergist trypsin inhibitors could potentially be developed to restore efficacy of Bt crops in controlling *H. zea* (Correy et al., 2019).

Gene duplications, an important source of genetic variation for phenotypic evolution, have been linked to many cases of insecticide and herbicide resistance evolution (Bass & Field, 2011; Heckel, 2022). Duplications of detoxification related genes can immediately confer resistance by increasing the quantity of already functional detoxification enzymes or can additionally lead to new mechanisms of resistance by neofunctionalization. Multiple cases of insecticide resistance have been functionally linked to new duplications of cytochrome P450s and carboxylesterases, two detoxification related gene families (Bass et al., 2013; Cattel et al., 2021; Mouches et al., 1990; Puinean et al., 2010; Schmidt et al., 2010). Our findings here suggest a new example of the duplication of a detoxification related gene leading to insecticide resistance, and the first case of this mechanism linked to Bt resistance. It is possible that gene duplication may be more likely to underlie rapid phenotypic evolution than other variant types, but the prevalence of structural variants is not well described as detection is difficult with current technologies (Ho et al., 2020; Mahmoud et al., 2019). As gene amplifications have frequently been linked to resistance evolution, genomic monitoring for resistance should be extended to consider a range of genomic variant types beyond SNPs (Fritz 2022).

## Methods

### Insect samples and phenotypes

Field samples of Cry resistant *H. zea*, defined as surviving to late instar on a Bt corn plant expressing Cry1A.105 + Cry2Ab2, were collected in 2019 and 2020 in Prince George's County (MD) at the University of Maryland CMREC farm in Beltsville. Late instars were collected from Cry1A.105 + Cry2Ab2 expressing sweet corn and reared in the lab on a 16:8 long day light cycle at 25°C and 50% room humidity. Newly emerged adults from field collections were single pair mated to produce a second generation of resistant field derived *H. zea*. Susceptible *H. zea* were acquired from a population that has been maintained in the laboratory without exposure to Cry toxins at Benzon Research (Cumberland County, PA). Cry tolerance in both the resistant field and susceptible lab populations was measured with a diagnostic dose in a corn leaf tissue incorporation assay as described in Taylor et al. (2021) to confirm resistant and susceptible status. Briefly, early second instar caterpillars were fed for seven days on a laboratory diet mixed with powdered lyophilized leaf tissue from Bt expressing corn and their non-Bt expressing near isolines. Resistance was measured by weight gain after seven days on Bt expressing leaf diets. Weight gain on the non-expressing near isoline control diets served as a measure of non-resistance associated growth.

The field derived progeny that grew well in the Cry expressing resistance assay (not in the bottom quartile for growth) were single pair mated to a susceptible individual from the lab colony. The specific susceptible individuals used in crosses were not exposed to the Cry toxin resistance assay due to the significant growth suppression in this highly susceptible population. Offspring of the single pair matings between the resistant and susceptible populations were sibling mated to produce an intercross $F_2$ generation. Cry toxin resistance phenotypes were

assessed in the $F_2$ progeny also using the leaf tissue incorporation assay, as described in Taylor et al. (2021). Five $F_2$ intercross families were assessed for resistance to Cry1Ab by measuring growth on diet with incorporated powdered Cry1Ab expressing leaf tissue. The other half of the offspring were assayed for growth related advantages on the diets with powdered leaf tissue from the non-expressing near isoline of Cry1Ab expressing corn. Five different intercross families were assessed for resistance to Cry1A.105 + Cry2Ab2 by measuring growth on diet with leaf tissue expressing both toxins for half of the offspring. The other half of the offspring were used to assess growth related advantages on diets containing leaf tissue from the non-expressing near isoline. Between 146 and 282 $F_2$ offspring were split across treatments and phenotyped from each family, resulting in 73 to 142 $F_2$ offspring phenotyped per treatment from each family. $F_2$ numbers varied due to differences in larval availability for assays. After phenotyping, the larvae were grown on a non-expressing lab diet to increase in size before DNA isolation. All samples were flash frozen and stored at -80°C.

The impact of experimental treatment and population on weight phenotypes after seven days of growth in the laboratory assays was assessed using a model reduction approach between nested general linear models all with a gamma distribution, as is appropriate for the continuous and positive weight phenotypes (Bolker, 2008). The treatments compared were 1) Cry1Ab expressing corn leaf tissue, 2) control tissue from the non-expressing near isoline of Cry1Ab corn, 3) Cry1A.105 + Cry2Ab2 expressing corn leaf tissue, and 4) control tissue from the non-expressing near isoline for Cry1A.105 + Cry2Ab2 corn. The populations compared were 1) the resistant field derived $F_0$, 2) susceptible lab colony $F_0$, and 3) the intercross $F_2$ offspring. The models were compared using a likelihood ratio $\chi^2$ test with an *a priori* $\boldsymbol{\alpha}$ of 0.01. Post hoc

470 pairwise contrasts were calculated with the R package emmeans (v. 1.8.6; Lenth et al., 2023)
471 with a bonferroni correction to p-values.

472 **DNA extraction and sequencing**

473 Individuals in the top and bottom 20% of weight phenotypes from each treatment and
474 family were selected for DNA extraction and sequencing. When individuals in the top or bottom
475 20% did not survive to preservation, they were replaced by individuals in the top or bottom 30%.
476 Genomic DNA was extracted from 833 high and low weight intercross $F_2$ offspring and all $F_1$
477 and $F_0$ parents with a DNeasy (Qiagen, Hilden, Germany) extraction kit using the modified
478 mouse tail protocol of Fritz et al. (2020). Larval and pupal tissue from the rear ½ - ⅓ of the
479 insect was used for extraction, while for adults, ½ of the thorax was used. DdRAD libraries were
480 prepared for all $F_2$ samples as described in Fritz et al. (2016, 2018). Briefly, samples were
481 digested with the restriction enzymes EcoRI and MSPI (New England Biolabs, Ipswich, MA),
482 and unique 6-mer or 8-mer barcodes were annealed prior to pooling with 38 - 39 other samples.
483 Pools were size-selected for 450-650 bp fragments using a Pippin Prep (Sage Scientific, Beverly,
484 MA), then 12 replicated PCR reactions with 14 cycles each were used to amplify DNA and add a
485 standard Illumina TruSeq index as an identifier to each pool. Pools were sequenced at North
486 Carolina State Genomic Sciences Laboratory on an S4 flow cell of the Illumina NovaSeq 6000,
487 resulting in 173.9 - 97.5 million raw 150 bp paired-end reads per sample pool with an average of
488 128 million reads per pool. Additionally whole genome sequencing was performed for the 20
489 cross founding parents on part of an S4 flow cell of an Illumina NovaSeq 6000 at the University
490 of Maryland Baltimore Institute for Genome Science. This sequencing resulted in 30.9 - 48
491 million raw reads per sample with an average of 39.5 million raw reads.

492 **Bioinformatic analysis**

493 $F_2$ ddRAD sequencing reads were demultiplexed and quality controlled using the stacks
494 script process_radtags (v.2.61; Rochette et al., 2019). The reads were only retained if they had
495 exact matches to the correct barcode sequence, quality scores above 20 in all 15 bp length sliding
496 windows, and no evidence of adapter contamination allowing up to 2 bp mismatch in adapter
497 sequence. Quality controlled reads were aligned to a *H. zea* chromosome scale assembly (v. 1.0,
498 PRJNA767434; Benowitz et al., 2022) with Bowtie (v.2.2.5; Langmead & Salzberg, 2012) using
499 the "very sensitive" alignment option. Variants were called with bcftools mpileup (v. 1.9;
500 Danecek et al., 2016) and pruned to include biallelic SNPs with a minor allele frequency > 0.05,
501 with a quality score > 50, present in > 50% of samples. The average sequencing coverage per
502 individual at a called ddRAD locus in the final variant set was 63×. The total number of ddRAD
503 sequencing markers remaining for genotype phenotype association ranged from 78,580 - 79,408
504 (Cry1Ab treatment group = 79,261, Cry1Ab control group = 79,182, Cry1A.105 + Cry2Ab2
505 treatment group = 79,408, Cry1A.105 + Cry2Ab2 control group = 78,580).

506 Whole genome sequencing reads were quality controlled with Trimmomatic (v. 0.39;
507 Bolgar et al 2014), to remove Illumina adapters, low quality regions at the beginning or end of
508 the read or where the average quality of a four base pair window fell below 30, and any reads
509 shorter than 36 bp. Trimmed reads were aligned and variants were called as described above for
510 the ddRADseq reads. In the filtered WGS data, a total of 4,716,009 high quality SNPs were
511 identified between resistant and susceptible populations, with a genome wide average sequencing
512 coverage of 18.6× (*st. dev.* = 2.4). For those analyses of the ddRAD sequencing data that
513 identified the direction of allele effect, SNPs were further filtered to include only those variants
514 where the allele origin (resistant or susceptible population) could be predicted with some

515 certainty. To identify SNPs where the likely origin population of the allele could be predicted,
516 only those SNPs with an allele count difference > 15 (out of a possible 20 alleles in each
517 population) between the resistant and susceptible parent populations were used. The population
518 where the allele was more common was used to identify the allele as resistance or susceptibility
519 associated. After filtering for effect direction, 6,717 - 6,749 SNPs remained (Cry1Ab treatment
520 group = 6,737, Cry1Ab control group = 6,749, Cry1A.105 + Cry2Ab2 treatment group = 6,733,
521 Cry1A.105 + Cry2Ab2 control group = 6,717).

522 **Resistant and susceptible founding populations genome wide comparison**

523 Genome-wide patterns of differentiation between the founding resistant and susceptible
524 populations were described using the variants identified by whole genome sequencing of the ten
525 field resistant and ten laboratory susceptible $F_0$ individuals founding the family crosses. A
526 principal components analysis was performed with Plink (v. 1.90b; Chang et al., 2015).
527 Divergence was measured by Weir and Cockerham's windowed $F_{ST}$ across 40kb windows with a
528 10kb step using VCFtools (v. 0.1.17; Danecek et al., 2011). A genome wide $zF_{ST}$ significance
529 threshold of 6 was used (Rubin et al., 2010). Downstream analysis of this data and all other data
530 presented here were performed in R (v.4.2.1; (R Development Core Team, 2023) with tidyverse
531 R package (v.2.0.0; Wickham et al., 2019), and visualizations were made with the R package
532 ggplot2 (v.3.4.1; Wickham, 2016)

533 **Quantitative trait mapping for Cry resistance**

534 Cry resistance genomic architecture was characterized by Bayesian sparse linear mixed
535 models (BSLMM) in gemma (v. 0.98.4; Zhou et al., 2013). For each treatment the model was run
536 five separate times, each time with a total of 5 million sampling steps with the first 500 thousand

discarded as burn-in. Hyperparameter estimates for each treatment were averaged across all five runs. The association between SNPs across the genome and weight phenotype from the resistance and control growth assays were detected with linear mixed models (LMM) also in gemma (v. 0.98.4; Zhou & Stephens, 2012). Both the BSLMM and LMM account for relatedness among the five replicated families for each treatment.

**Differential gene expression analysis**

In 2022, Cry1A.105 + Cry2Ab2 expressing corn ears and ears from the non-Bt expressing near isoline were collected from the University of Maryland CMREC farm at Beltsville. Ears that were infested with *H. zea* were identified by the loosened silk and brought to the laboratory. In the lab, each corn ear was open and any 5th instar larvae present were identified and removed from the ear; caterpillars at other developmental stages were not included in the experiment. Immediately following removal, larvae were chilled and immobilized in a 70% solution of ice cold RNAlater and PBS. The head capsule on each larva was removed and stored for later measurement to confirm instar. All larvae were later confirmed as 5th instar by head measurements between 1.60 to 2.70 mm (Bilbo et al., 2019). The midgut was cut away from the crop and intestine, and the malpighian tubules were removed. Corn contents was cleared from midgut using forceps and a rinse of 70% RNAlater. Midguts were flash frozen and stored at -80°C. The dissection process was repeated with individuals from the laboratory susceptible colony, which had been reared on a standard laboratory diet not containing any corn tissue or Bt toxins.

Midguts of 5th instars from Bt expressing corn, non-Bt corn and the susceptible laboratory colony were organized into eight randomized pools per treatment with four samples in each pool (n = 32 per treatment). RNA was extracted from each pool using a Qiagen RNeasy®

560 Mini Kit according to the manufacturer protocol except for the initial pooling of samples. Poly-A
561 selection, library preparation, and sequencing were completed by the University of Maryland
562 Baltimore Genomics Core Facility. All RNA libraries had a RIN value between 8.7 and 10, with
563 22/24 having RIN of 10, indicating very high quality. Libraries were 100 bp paired end
564 sequenced on an Illumina NovaSeq 6000.

565 Raw reads were quality controlled with Trimmomatic (v. 0.39; Bolger et al., 2014) to
566 remove Illumina adapters, trim reads where 4 bp sliding window quality score fell below 20, and
567 drop any reads shorter than 36 bp after trimming. The paired quality controlled reads were
568 aligned to a chromosome scale *H. zea* assembly (Benowitz et al., 2022; GCA_022343045.1) with
569 HISAT2 (v. 2.2.1; Kim et al., 2019) using default parameters. Gene annotations were lifted from
570 the older reference genome Hzea_1.0 (Pearce et al., 2017; GCA_002150865.1) to the new
571 chromosome scale assembly using Liftoff (v. 1.6.1; Shumate & Salzberg, 2021). Gene expression
572 counts were generated with HTSeq-count (v. 0.13.5; Anders et al., 2015). Expression patterns
573 across all 24 pools were compared through differential expression analysis in DESeq2 (v. 1.38.3;
574 Love et al., 2014). Two-way differential expression analysis was conducted between resistant
575 field individuals collected from Bt expressing corn and susceptible lab colony samples to
576 identify expression changes associated with resistance. An additional two-way differential
577 expression analysis between the field collected individuals from Bt expressing corn and non
578 expressing corn was conducted to identify Cry exposure inducible gene expression changes.
579 Statistical significance was indicated by a false discovery rate adjusted p-value of 0.01.

580 **Structural variant detection**

581 Sequencing coverage depth signals were extracted from aligned .bam files. The genome
582 wide coverage depth and the average coverage depth for the trypsin cluster were calculated with

583 Qualimap (v. 2.2.1; Okonechnikov et al., 2016), while windowed coverage depth calculations
584 were from samtools depth (v. 1.10; Danecek et al., 2021) results. The presence of the structural
585 variant in 2002, 2017, and 2019 was assessed using the publicly available whole genome
586 sequencing data from Taylor et al. (2021) (PRJNA751583).

587 The structural variant was validated with digital droplet PCR (ddPCR) for trypsin 77, one
588 of seven trypsins in the putative duplication. ATP dependent DNA helicase was used as a control
589 gene, as it is single copy in lepidoptera in OrthoDB (Zdobnov et al., 2021). Twenty-five ng of
590 DNA per sample was mixed with HindIII and analyzed in multiplexed assay for trypsin 77 and
591 ATP dependent DNA helicase at MOgene (St. Louis, MO). The genes were targeted with the
592 primers shown in **(Table S8)** and the following amplification conditions: activation at 95°C for
593 10 minutes, followed by 40 cycles of denaturing at 94°C for 30 seconds, annealing and extension
594 at 58°C for 1 minute, finally the enzyme was deactivated at 98°C for 10 minutes and held at 4°C.
595 ddPCR was performed with QX200 Automated Droplet Generator and Reader and analyzed
596 using the QX Manager 1.2 Standard Edition software (Bio-Rad, Hercules CA). All samples were
597 run with a synthesized gBlock positive control and a no template negative control. Copy number
598 for samples was calculated as follows:

$$CN = \frac{\ln\left(1 - \frac{\text{target gene positive droplets}}{\text{total number of droplets}}\right)}{\ln\left(1 - \frac{\text{control gene positive droplets}}{\text{total number of droplets}}\right)}$$

599

600 **Field resistant colony collection and rearing**

601 Putatively Cry resistant *H. zea* late instar caterpillars were collected from Cry1Ab
602 expressing sweet corn ('BC0805') ears grown at the Central Maryland Research and Education
603 Center in Beltsville, Maryland. Approximately 450 caterpillars were collected during each of two
604 collection dates (August 18 and September 7, 2023). Caterpillars were reared on *H. zea* diet

605 (Southland Products Inc., Arkansas, USA) until adulthood and bucket mated in a growth
606 chamber set at 25°C with 70% relative humidity and 16:8 light:dark cycle. Eggs from bucket
607 matings were placed onto diet and reared until the second instar under the same conditions.
608 Occasionally, larvae were held at 4°C once they reached the appropriate size to ensure sufficient
609 numbers at the same developmental stage for assays.

610 **Trypsin inhibition assay**

611 To determine whether trypsin activity impacts Cry toxicity in *H. zea*, we
612 compared 7-day larval growth in a diet incorporation corn leaf tissue bioassay with 12
613 treatments: 1) 1X TLCK trypsin inhibitor and Cry1Ab expressing leaf tissue, 2) 2X TLCK
614 trypsin inhibitor and Cry1Ab expressing leaf tissue, 3) 5X TLCK trypsin inhibitor and Cry1Ab
615 expressing leaf tissue, 4) 10X TLCK trypsin inhibitor and Cry1Ab expressing leaf tissue, 5) 1X
616 TLCK trypsin inhibitor and non-expressing leaf tissue, 6) 2X TLCK trypsin inhibitor and
617 non-expressing leaf tissue, 7) 5X TLCK trypsin inhibitor and non-expressing leaf tissue, 8) 10X
618 TLCK trypsin inhibitor and non-expressing leaf tissue, 9) buffer and Cry1Ab expressing leaf
619 tissue, 10) buffer and non-expressing leaf tissue, 11) Cry1Ab expressing leaf tissue alone, 12)
620 non-expressing leaf tissue alone. These treatments enabled us to separately determine the growth
621 effect of Cry expressing tissue, the buffer, and the inhibitor at different concentrations. Diet
622 bioassays were performed between June and November of 2023. Second instars from the
623 laboratory Cry susceptible *H. zea* population from Benzon research and field-collected Cry
624 resistant *H. zea* were reared on these treatments for seven days before they were individually
625 weighed to assess growth. In each assay 8 - 16 individuals were exposed to each treatment, due
626 to availability of appropriately sized larvae. Final sample sizes were ≥ 70 in each treatment, and
627 are reported in Table S6.

Based upon Ma et al. (2013) and preliminary trials, we selected N-α-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK, Sigma-Aldrich®, St. Louis, MO, USA) as our trypsin inhibitor. To make the inhibitor solutions, 350 mg of TLCK was dissolved in 10 mL of phosphate buffer solution (0.1M, pH 5.8, bioWORLD, Dublin, OH, USA) to make a stock solution of 35 mg/mL (10X). This solution was further diluted to 3.5 mg/mL (1X), 7 mg/mL (2X), and 17.5 mg/mL (5X) as needed. The inhibitor solutions were freshly made on each date. A 10 mL aliquot of phosphate buffer was used for the buffer control treatment. The bioassays were prepared as in Dively (2016), Taylor et al. (2021), and as described above, except, in the inhibitor and buffer treatments 1.6 mL of the TLCK solution or phosphate buffer were mixed into 25 mL of experimental diet, resulting in the following final TLCK concentrations: 1X: 224.5 µg of TLCK per mL diet, 2X: 449 µg of TLCK per mL diet, 5X: 1.1 mg of TLCK per mL diet, 10X: 2.2 mg of TLCK per mL diet.

First we compared the two control groups (incorporated leaf tissue only and incorporated leaf tissue with buffer) using t tests with an alpha of 0.01 to identify any potential effects of the buffer alone. Then, we tested for a synergistic effect of trypsin inhibition and Cry exposure on growth in *H. zea* with a general linear model comparison approach. A gamma distribution was used for the weight response variable. The fit of the models was compared with a likelihood ratio chi-squared test using an alpha of 0.01. We tested for combined effects by comparing a full model with Cry treatment and trypsin inhibition main effects and an interaction between the two, to a model with only the two main effects. Bonferroni corrected post hoc contrasts were calculated with the R package emmeans (v. 1.8.6; Lenth et al., 2023). We also calculated a growth ratio at each TLCK dose as:

$$GR = \frac{weight\ on\ Cry\ expressing\ leaf\ tissue\ at\ TLCK\ dose\ x}{weight\ on\ the\ non-expressing\ leaf\ tissue\ at\ TLCK\ dose\ x}$$

The growth ratio measured the combined effect of TLCK and Cry toxins, and would be expected to stay consistent across doses if the effects of TLCK and Cry toxins on growth suppression are not linked.

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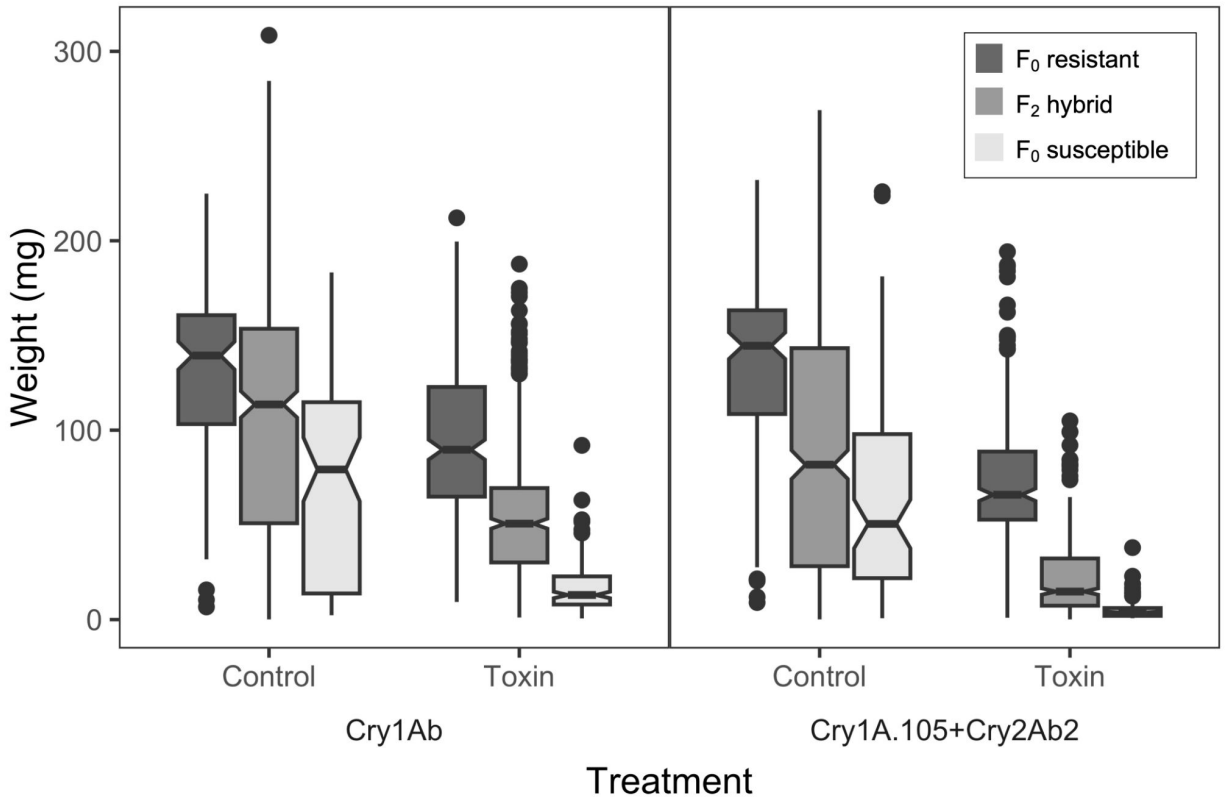
682 **Figures**




683

684 **Figure 1.** Distribution of weights for *H. zea* individuals after seven days in a laboratory leaf
685 tissue incorporation assay for Cry toxin and control treatments.
686
687

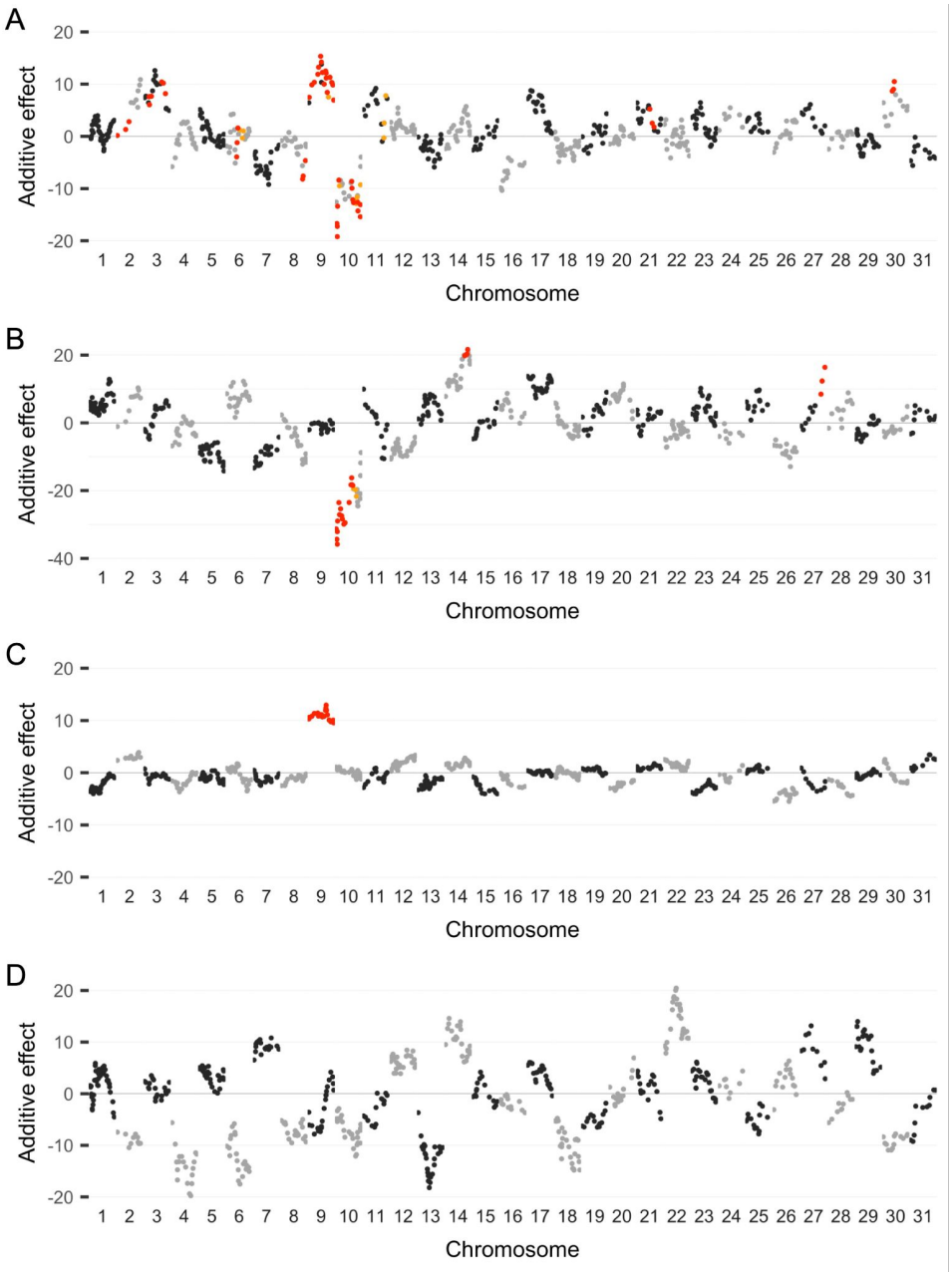



**Figure 2.** Association of genome wide markers and weight gain after seven days of feeding on Cry toxin containing and control treatments. The additive effect (beta from LMM) is the weight gain in mg associated with the presence of a single resistant population allele smoothed for visualization by averaging over 21 SNP windows with a 5 SNP step for markers on 31 *H. zea* chromosomes. The genotype-phenotype association is shown for **A.** weight gain after seven days of exposure to Cry1Ab, **B.** weight gain on the non-toxin near isoline control diet for Cry1Ab, **C.** weight gain after seven days of exposure to Cry1A.105 + Cry2Ab2, **D.** weight gain on the non-toxin near isoline control diet for Cry1A.105 + Cry2Ab2. Chromosomes are plotted in alternating dark and light gray with each point representing a 21 SNP window. Windows with at least one significantly associated SNP are highlighted in color, with orange indicating an adjusted p value < 0.05 and red indicating an adjusted p value < 0.01.

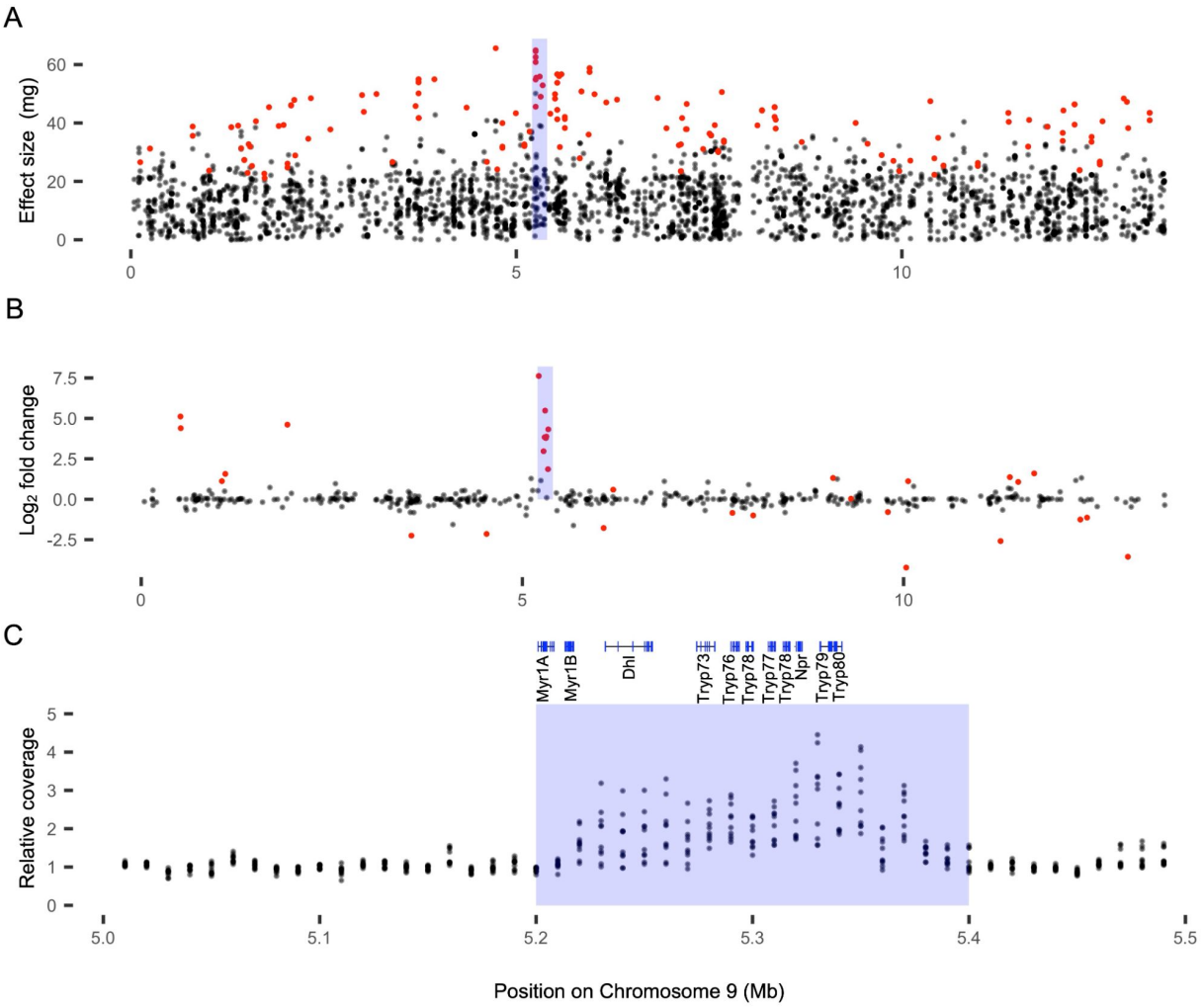



**Figure 3.** Genomic signals suggesting that a region between 5.2 and 5.4 Mb on *H. zea* Chr 9 is duplicated in field resistant individuals and associated with resistance phenotype. The absolute value of the effect size (beta from the LMM) for ddRAD SNPs is plotted in (**A**). A differential expression plot for all genes on Chr 9 between resistant and susceptible populations is shown in (**B**). In (**C**) the gene annotations between 5.2 and 5.4 Mb on Chr 9 are shown above a plot of relative sequencing coverage for field resistant *H. zea*. Gene abbreviations are as follows: Myr1 = myrosinase 1-like, Dlh = disks large homolog 4-like, Tryp = Trypsin, Npr = neuropeptide receptor A35. In all panels the region between 5.2 and 5.4 Mb on Chr 9 is highlighted in blue.

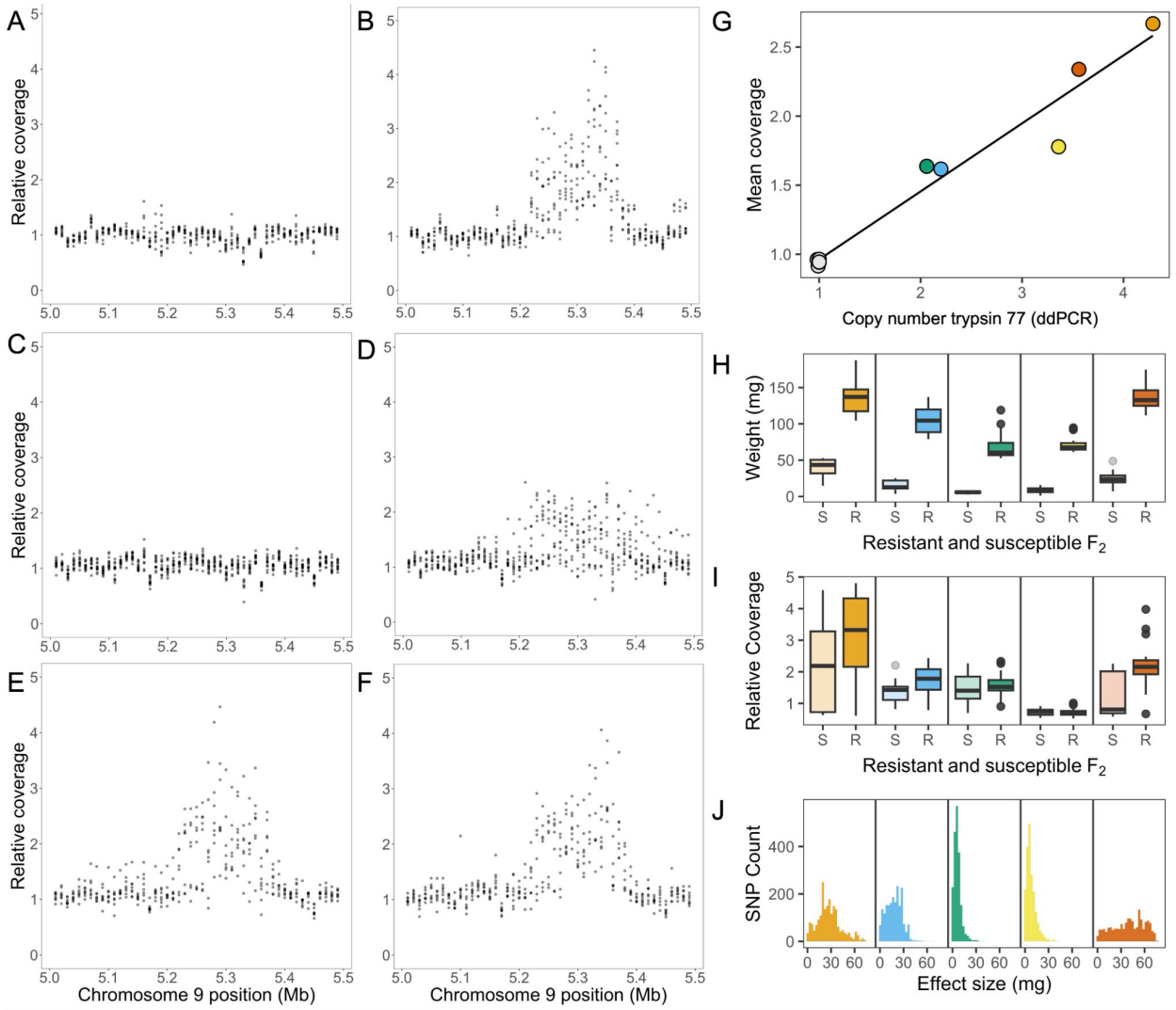



**Figure 4**. Gene amplification detection on *H. zea* Chr 9 from 5 - 5.5 Mb. In A - F each point represents the average sequencing depth in that 10 kb window for a single individual relative to that individual's genome wide average sequencing coverage. Relative sequencing depth is plotted in **(A)** susceptible and **(B)** resistant founding cross parents, **(C)** individuals collected in 2002 before Cry resistance evolution and **(D)** 2017 after practical resistance was first detected, and **(E)** individuals collected in 2019 in non-Bt expressing corn and **(F)** Bt expressing corn. Coverage signals are associated with ddPCR copy number variant analysis for Trypsin 77 ($r^2 = 0.95$) for the found resistant (mixed colors) and susceptible (gray) parents of mapping families **(G)**. Across the 5 replicated Cry1Ab resistance mapping families (in the same color as the founding parent in G), the weight **(H)** and ddRAD coverage depth of the trypsin cluster **(I)** of the most susceptible (S) and resistant (R) $F_2$ offspring are shown, along with a histogram of Cry1Ab resistance effect sizes for all Chromosome 9 SNPs **(J)**.

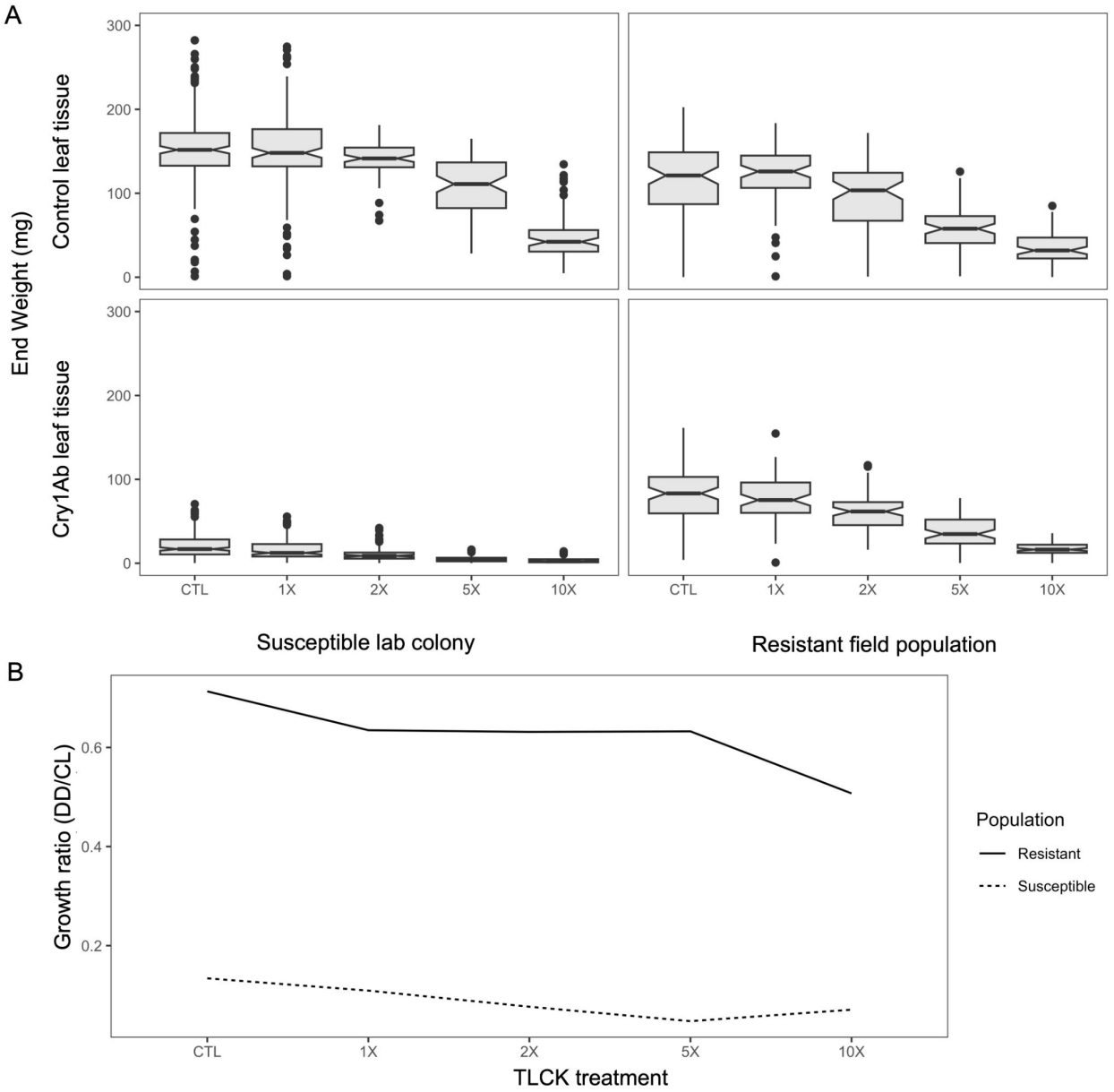



**Figure 5.** Distribution of weights for laboratory susceptible *H. zea* individuals after seven days in a trypsin inhibition assay with Cry1Ab containing corn leaf tissue and leaf tissue from the non-expressing near isoline.

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