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Research Article



# Quantitative Trait Loci Mapping of Western Corn Rootworm (Coleoptera: Chrysomelidae) Host Plant Resistance in Two Populations of Doubled Haploid Lines in Maize (*Zea mays* L.)

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## Abstract

Over the last 70 yr, more than 12,000 maize accessions have been screened for their level of resistance to western corn rootworm, *Diabrotica virgifera virgifera* (LeConte; Coleoptera: Chrysomelidae), larval feeding. Less than 1% of this germplasm was selected for initiating recurrent selection or other breeding programs. Selected genotypes were mostly characterized by large root systems and superior root regrowth after root damage caused by western corn rootworm larvae. However, no hybrids claiming native (i.e., host plant) resistance to western corn rootworm larval feeding are currently commercially available. We investigated the genetic basis of western corn rootworm resistance in maize materials with improved levels of resistance using linkage disequilibrium mapping approaches. Two populations of topcrossed doubled haploid maize lines (DHLs) derived from crosses between resistant and susceptible maize lines were evaluated for their level of resistance in three to four different environments. For each DHL topcross an average root damage score was estimated and used for quantitative trait loci (QTL) analysis. We found genomic regions contributing to western corn rootworm resistance on all maize chromosomes, except for chromosome 4. Models fitting all QTL simultaneously explained about 30 to 50% of the genotypic variance for root damage scores in both mapping populations. Our findings confirm the complex genetic structure of host plant resistance against western corn rootworm larval feeding in maize. Interestingly, three of these QTL regions also carry genes involved in ascorbate biosynthesis, a key compound we hypothesize is involved in the expression of western corn rootworm resistance.

**Key words:** *Diabrotica virgifera virgifera*, host plant resistance, maize, QTL, insect resistance

Western corn rootworm, *Diabrotica virgifera virgifera* (LeConte; Coleoptera: Chrysomelidae), herbivory on maize causes an estimated annual loss of one billion dollars in the United States (Metcalf 1986). Root feeding injury caused by western corn rootworm larvae ranges from a few feeding scars and root tip injury to the destruction of entire root nodes. For each node that is destroyed, yield is reduced by approximately 15% (Tinsley et al. 2013). When drought is present, such as many areas in 2012, yield loss can be catastrophic (Tinsley et al. 2015). Western corn rootworm root feeding reduces the uptake of water and nutrients and also compromises plant stability (Levine and Oloumi-Sadeghi 1991). Lodged plants decrease light uptake of the canopy resulting in reduced photosynthesis efficiency, obstruct harvesting, and subsequently, lead to additional yield reduction (Levine and Oloumi-Sadeghi 1991). Current western corn rootworm management strategies rely heavily on crop rotation systems and genetically modified maize hybrids carrying *Cry* genes active against western corn rootworm larvae. Seed treatments with insecticides, soil insecticides at planting time, and adult management systems are additional methods to manage the western corn rootworm. However, western corn rootworm is known for its ability to overcome adverse conditions. They have developed resistance to soil insecticides (Ball and Weekman 1962), insecticides targeting beetles (Meinke et al. 1998, Pereira et al. 2015), Bt toxins (Gassmann et al. 2011, Zukoff et al. 2016), and even crop rotation by changing egg-laying behavior (Gray et al. 2009). Therefore, pest management systems integrating multigenic

host plant resistance could serve an important new role in managing the western corn rootworm.

A major roadblock towards improved host plant resistance of maize against western corn rootworm is the apparent lack of genetic variation for this trait in the Midwestern maize pools. Some progress has been made improving tolerance to western corn rootworm larval feeding from the 1960s to the 1980s and promising sources of resistance have been identified (Branson et al. 1983; Kahler et al. 1985; Assabgui et al. 1995; Hibbard et al. 1999, 2007; Flint-Garcia et al. 2009). However, no hybrids claiming native (i.e., host plant) resistance to western corn rootworm larval feeding are currently commercially available.

Typically, in breeding programs for western corn rootworm host plant resistance, screening has been done using inbred lines, populations, or landraces, but not as hybrids with elite inbreds (topcrosses) (Flint-Garcia et al. 2009). However, when a source of western corn rootworm host plant resistance was crossed to elite inbreds, the resulting topcrosses were less damaged than the resistant parent, even when the elite line used as the topcross parent was susceptible to rootworm feeding (Hibbard et al. 2007). Given that farmers grow hybrids, not inbreds or populations, breeders need to understand how inbreds perform in hybrid combinations. Flint-Garcia et al. (2009) observed that topcrossed inbreds tend to have reduced western corn rootworm damage. They evaluated 25 diverse inbred lines and their B73 hybrids for western corn rootworm damage in seven environments. Overall, hybrids had significantly less damage than inbreds, and more importantly, the correlation between inbreds and hybrids was not significant.

El Khishen et al. (2009) evaluated the resistance mechanism of maize genotypes with improved western corn rootworm resistance. They reported that this group of genotypes had less damage, fewer and smaller larvae recovered, and fewer adults recovered than the susceptible controls. These results demonstrate that improving western corn rootworm resistance using conventional breeding procedures is possible. However, a deeper understanding of the genetic basis of western corn rootworm resistance is needed to identify new sources of resistance rapidly and to combine these efficiently in agronomically acceptable maize cultivars.

The objectives of our study were to 1) map quantitative trait loci (QTL) conferring resistance to western corn rootworm larvae feeding in two populations of topcrossed doubled haploid maize lines (DHLs) derived from crosses between susceptible and resistant maize inbreds adapted to the U.S. Corn Belt, 2) estimate their genetic effects, and 3) investigate the consistency of western corn rootworm resistance QTL across mapping populations.

## Materials and Methods

### Plant Materials

Western corn rootworm resistant maize lines CRW8-1 and NGSDCRW1-15-2S2 and conventional elite but western corn rootworm susceptible maize inbreds AG1 and LH51 were used as parents. The CRW8-1 line was derived from the BS20 (S)C2 synthetic registered by Russell et al. (1976). The NGSDCRW1-15-2S2 (shortened to NGSDCRW1 hereon) was derived from the remnant seed of the breeding program from which NGSDCRW1(S2)C4 was released (Kahler et al. 1985). Both were identified as being resistant in the USDA-ARS breeding program in Columbia, Missouri. Two $F_1$ hybrids derived from crosses between resistant and susceptible parents (NGSDCRW1×AG1 and LH51×CRW8-1) were used to produce populations of DHLs. All DHLs and their parents were topcrossed with susceptible inbred PHZ51. The initial crosses and the production of DHLs and their topcrosses were conducted by AgReliant Genetics, LLC., in Illinois and Puerto Rico in 2007 and 2008. A total of 142 DHLs were available for population NGSDCRW1×AG1 and 134 DHLs for population LH51×CRW8-1. Also, we tested the parental inbreds *per se* and as hybrids crossed to tester PHZ51. An AgReliant commercial Cry3Bb1-expressing Bt hybrid was used as the resistant check. We used the non-Bt version of the commercial hybrid and hybrid B37×H84 as susceptible checks. B37×H84 is highly susceptible to corn rootworm larval feeding (El Khishen et al. 2009). As a supplementary entry, B37×H84 was protected by the insecticide Force 3G (Syngenta Crop Protection Inc., Greensboro NC).

### Experimental Design

Experiments were conducted in 2009 at the Bradford Research and Extension Center and the Poultry Farm both in Columbia, MO, the University of Illinois Research and Education Center in Urbana, IL, and at the USDA-ARS Eastern South Dakota Soil and Water Research Farm in Brookings, SD.

The experimental design for population NGSDCRW1×AG1 was a 26 × 6 α design and for population LH51×CRW8-1 a 25 × 6 α design both with three replications at each location. In Columbia, MO, nine seeds were planted in 1.83 m single row plots using a research planter designed for short plots. In Brookings and Urbana, ten seeds were planted in 2.3 m-long single row plots. Both artificial and natural infestations were used to create western corn rootworm pressure. Western corn rootworm eggs were provided by the USDA-ARS North Central Agricultural Research Laboratory in Brookings, SD, suspended in 0.15% agar solution (Palmer et al. 1977), and mechanically placed 8–10 cm into the soil using techniques modified from Sutter and Branson (1980). In Columbia, western corn rootworm eggs were mechanically applied when plants were at the V2 stage of development. The Bradford and Poultry Farm locations were infested with 1,000 eggs per 30.5 cm of row. At Brookings, plots were mechanically infested at a rate of 900 eggs per 30.5 cm immediately before hand planting. Trap crop enhanced natural infestation was employed as the sole method at the Urbana location.

Root damage was assessed after the majority of western corn rootworm larvae had stopped feeding and entered the pupal stage. Rootworm development was determined by monitoring soil growing degree days (Fisher et al. 1990), and digging up plants from infested check plots. Four randomly selected plants were processed per plot in Missouri and South Dakota. Five plants per plot were processed in Urbana to account for the larger variation in the spatial distribution of western corn rootworm eggs in trap crop experiments. Plants were cut at 0.5 m height and roots dug with potato forks or spades in Brookings and Urbana, whereas in Columbia, roots were excavated using a specially-designed tractor-mounted implement (Praiswater et al. 1997). Roots were soaked in water-filled tanks and washed using high-pressure sprayers. Roots were then rated for rootworm damage (RDR) using the 0 to 3 scale (Oleson et al. 2005). This linear rating scale uses the following criteria: 0 = no root damage, 1 = one node of roots eaten, 2 = two nodes eaten, 3 = three nodes eaten. Root size (RSZ) and compensatory root growth (RRG) were evaluated using 1 to 6 categorical scales (RSZ: 1 = largest, 6 = smallest; RRG: 1 = most, 6 = none; Rogers et al. 1977).

### Phenotypic Data Analysis

Analyses of variance were performed for all traits using plot means applying the following statistical model:

$$y_{ijklm} = \mu + \alpha_i + \beta_{(i)j} + \delta_{(ij)k} + \varphi_l + (\varphi\alpha)_{il} + \varepsilon_{ijklm} \quad (1)$$

Where, $y_{ijklm}$ denotes the plot mean of genotype $l$, $\mu$ is the overall mean, $\alpha_i$ is the random effect of the $i^{th}$ location, $\beta_{(i)j}$ is the random effect of the $j^{th}$ replication in the $i^{th}$ location, $\delta_{(ij)k}$ is the random effect of the $k^{th}$ block in the $j^{th}$ replication of the $i^{th}$ location, $\varphi_l$ is the random effect of the $l^{th}$ genotype, $(\varphi\alpha)_{il}$ is the random effect of the $l^{th}$ genotype by the $j^{th}$ location interaction, and $\varepsilon_{ijklm}$ represents the random residual error. Estimates of variance components $\sigma^2_{error}$ (error variance), $\sigma^2_{ge}$ (genotype × environment interaction variance), and $\sigma^2_g$ (genotypic variance) of DHL topcrosses were used to calculate broad-sense heritabilities as $H^2 = \sigma^2_g / (\sigma^2_g + \frac{\sigma^2_{ge}}{l} + \frac{\sigma^2_{error}}{lr})$, with $l$ and $r$ being the number of locations and replications within each location, respectively. Standard errors for $H^2$ estimates were calculated using the square root of the variance estimate for $H^2$ (Holland et al. 2003). The best linear unbiased estimate (BLUE) for the $l^{th}$ genotype was computed regarding genotypes in equation 1 as a fixed effect and used for QTL mapping. Multivariate analysis of variance models were applied to calculate phenotypic ( $r_P$ ) and genotypic ( $r_g$ ) correlation coefficients between traits and to determine their significance (Holland 2006). All analyses were performed with SAS statistical software 9.2 (SAS Institute 2011) procedure PROC MIXED.

### Segregation and Linkage Analyses

All parental inbreds and DHLs were genotyped with 768 single nucleotide polymorphism (SNP) markers by AgReliant on Illumina Golden Gate chips. The significance of the deviation of each molecular marker class from expected Mendelian segregation (1:1) and observed allele frequencies for deviation from the expected allele frequency of 0.5 was determined using standard chi-square tests. Appropriate Type I error rates were determined by the sequentially rejective Bonferroni procedure (Holm 1979) to account for the large number of tests conducted. The level of heterozygosity (%) of the parental inbred lines and of each DHL was estimated by dividing the observed number of heterozygous marker loci by the total number of scorable marker loci in the respective DHL. For population NGSDCRW1×AG1, a linkage map was constructed based on 141 DHLs and a total of 179 polymorphic molecular markers. For population LH51×CRW8-1, 134 DHLs were used to map 173 polymorphic markers. Both linkage maps were built using the software package GMendel (Liu and Knapp 1990). Linkage between two markers was declared significant in the two-point analyses when the LOD score ($\log_{10}$ of the likelihood odds ratio) exceeded the threshold of 3.0. After determination of linkage groups and the correct linear arrangement of marker loci along the chromosomes, recombination frequencies between marker loci were estimated by multipoint analyses and transformed into centiMorgan (cM) by Haldane's (1919) mapping function.

### QTL Analysis

QTL mapping was based on DHL topcross BLUEs for all three root phenotypic trait. The method of composite interval mapping was employed for QTL detection and estimation of their effects in Windows QTL Cartographer 2.5 (Wang et al. 2007). Cofactors were selected applying the backward regression method. All detected QTL were fit simultaneously in one model to estimate their effects and epistatic interactions as well as their partial contribution to the total phenotypic variance. The significance of QTL×Environment (QTL×E) interactions was determined as described by Bohn et al. (1996) and implemented in PlabQTL (Utz and Melchinger 1996). If traits showed a high genotypic correlation ($r_g > 0.5$), we conducted a multi-trait analysis using the joint composite interval mapping method developed by Jiang and Zeng (1995) and implemented in the JZmapqtl option of Windows QTL Cartographer 2.5. The joint composite interval mapping method enabled us to test the genetic hypothesis of pleiotropy versus linkage of QTL identified for multiple traits. For each trait and multi-trait combination, mapping population specific empirical genome-wide LOD thresholds were determined using 1,000 permutations.

## Results

### Segregation and Linkage of SNPs

Large marker intervals that span a distance of more than 50 cM were found for mapping population NGSDCRW1×AG1 on all chromosomes except C9 and mapping population LH51×CRW8-1 on chromosomes C1, C3, C4, C6, C7, and C8 (see Supplementary Information A—Linkage Maps). Therefore, two to four partial linkage groups per chromosome were found. However, the mapping data supported combining these partial linkage groups to one linkage group in accordance with published maps (Andorf et al. 2010). Including all marker intervals with LOD > 2.0, the linkage map constructed for cross NGSDCRW1×AG1 contained 176 molecular markers, which span a total distance of 1863 cM with an average interval length of 11.2 cM. For cross LH51×CRW8-1 173 markers span a total distance of 2247 cM with an average marker distance of 13.8 cM.

### Agronomic Trait Analysis

#### The Performance of Check Hybrids

The same check hybrids were used in the evaluation of both mapping populations and performed as expected. The Bt hybrid showed no root feeding damage (RDR < 0.08), only a small amount of root regrowth (RRG > 5.15) and the largest root system (RSZ < 2.27). The non-Bt near-isoline hybrid was significantly ($P < 0.05$) more damaged by western corn rootworm larvae (RDR > 1.00) than the Bt-hybrid. As a consequence, the non-Bt hybrid also displayed more root regrowth (RRG > 4.22) and a smaller root size (RSZ > 2.91). Root damage ratings for susceptible check hybrid B37×H84 were high (RDR > 1.71). When B37×H84 was treated with an insecticide, the RDR values were not significantly ($P < 0.05$) different from the Bt-hybrid. Root regrowth and RSZ were not different for B37×H84 treated with or without insecticide. The RDR difference between Bt hybrid and insecticide treatment were not significant in both experiments.

#### Topcross Performance of NGSDCRW1×AG1

The average topcross performance of NGSDCRW1×AG1 derived DHL topcross populations for RDR, RRG, and RSZ was 1.22, 4.67, and 3.36, respectively (Table 1). For RDR, DHL topcross means ranged from 0.70 to 1.73, not exceeding the RDR values observed for check hybrid B37×H84 grown with (RDR = 0.40) and without (RDR = 1.91) insecticide protection. The average RDR of the DHL topcross population was not significantly different from the mean RDR of the non-Bt check hybrid. Root regrowth ranged from 4.05 to 5.35 with an average RRG of 4.67. Root size varied between 2.69 and 4.29 with an average RSZ of 3.36. Genotypic variances ( $\hat{\sigma}^2_g$ ) were significant ($P < 0.05$) or highly significant ($P < 0.01$) for all traits. Estimates of $\hat{\sigma}^2_{ge}$ were also highly significant ($P < 0.01$) for RDR and RRG but not significant for RSZ. Due to significant

**Table 1.** Means of parents NGSDCRW1 and AG1, and 142 Doubled Haploid Lines derived from their cross and evaluated as topcrosses and estimates of variance components and heritabilities among DHL topcrosses for root damage caused by western corn rootworm feeding, root regrowth, and root size evaluated in three environments

| Statistics | Entries | Traits | | |
|---|---|---|---|---|
| | | Root damage | Root regrowth | Root size |
| | | 0–3 scale | 1–6 scale[a] | 1–6 scale[b] |
| Means | | | | |
| NGSDCRW1 | 1 | 2.33 ± 0.16[c] | 5.62 ± 0.21 | 4.44 ± 0.18 |
| NGSDCRW1-TC | 1 | 1.05 ± 0.17 | 4.19 ± 0.22 | 3.33 ± 0.18 |
| AG1 | 1 | 0.48 ± 0.16 | 5.49 ± 0.21 | 4.84 ± 0.18 |
| AG1-TC | 1 | -/- | -/- | -/- |
| DHL-TC | 142 | 1.22 ± 0.22 | 4.67 ± 0.28 | 3.36 ± 0.23 |
| Check hybrids | | | | |
| Bt-Hybrid | | 0.08 ± 0.17 | 5.09 ± 0.22 | 2.77 ± 0.18 |
| Non-Bt Iso hybrid | | 1.04 ± 0.17 | 4.22 ± 0.22 | 3.48 ± 0.18 |
| B37×H84 insecticide | | 0.40 ± 0.22 | 5.05 ± 0.23 | 3.77 ± 0.18 |
| B37×H84 | | 1.91 ± 0.17 | 5.59 ± 0.22 | 3.89 ± 0.18 |
| Variance components (DHL topcrosses) | | | | |
| $\sigma^2_g$ | | 0.0160* | 0.0149* | 0.0190** |
| $\sigma^2_{ge}$ | | 0.0339** | 0.0369** | 0.0073[ns] |
| $\sigma^2_{error}$ | | 0.2086** | 0.3167** | 0.3418** |
| Broad-sense heritability[c] | | 0.39 ± 0.11 | 0.31 ± 0.10 | 0.39 ± 0.09 |

[a]Root regrowth was assessed using a rating scale from 1 (a large amount of regrowth) to 6 (no regrowth).
[b]Root size was assessed using a rating scale from 1 (large root) to 6 (small root).
[c]SEs are attached.
*,**Significant at the 0.05 and 0.01 probability levels, respectively; ns, nonsignificant.

genotype × environment interactions and large error variances, heritabilities were low for all traits ranging from 0.31 for RRG to 0.39 for RDR and RSZ. Phenotypic correlation coefficients were positive and highly significant ($P < 0.01$) but less than 0.45 for all trait pairs (Table 3). Only RDR and RSZ showed a significant genotypic correlation (Table 3).

### Topcross Performance of LH51×CRW8-1

The average topcross performance of LH51×CRW8-1 derived DHL topcrosses for RDR, RRG, and RSZ was 1.57, 5.15, and 3.31, respectively (Table 2). For RDR, DHL topcross means ranged from 0.70 to 2.28 exceeding the RDR mean of the susceptible check hybrid B37×H84 (RDR = 1.72). The average RDR of the LH51×CRW8-1 derived DHL topcross population was not significantly different from the mean RDR of the susceptible check. Root regrowth ratings ranged from 4.41 to 5.73 with an average RRG of 5.15. Root size varied between 2.72 and 3.85 with an average RSZ of 3.31. Genotypic variances and $\hat{\sigma}^2_{ge}$ were significant ($P < 0.05$) or highly significant ($P < 0.01$) for all traits. Heritabilities were low for all traits ranging from 0.28 for RSZ to 0.43 for RDR. Phenotypic and genotypic correlation coefficients among traits were positive and highly significant ($P > 0.01$) but less than 0.49 for most trait combination (Table 3). Exceptions were RDR and RSZ with $r_p = 0.59$ and $r_g = 0.91$.

## QTL Analyses

### NGSDCRW1×AG1

Five putative QTL affecting RDR were detected on chromosomes 2, 7, 9, and 10 explaining between 5.6% and 14.8% of $\sigma^2_p$, with LOD values ranging from 3.04 to 7.85 (Table 4). Alleles increasing the level of resistance (i.e., decreasing RDR) were contributed by both parental inbreds. A simultaneous fit with all five putative QTL explained 29.2% of $\sigma^2_g$. QTL×environment interactions were significant ($P < 0.01$) for all QTL except for the QTL on chromosome 2. For RRG, QTL on chromosomes 1, 2, 6, 8, and 9 explained a total of 31.9% of $\sigma^2_g$. All QTL alleles reducing RRG originated from parental inbred NGSDCRW1. Significant QTL×E interactions were detected for RRG QTL on chromosome 6 ($P < 0.01$). Three QTL were detected for RSZ on chromosomes 1 (two QTL) and 10 and explained 24.2% of $\sigma^2_g$. Only the QTL on chromosome 10 showed a significant ($P < 0.05$) interaction with environments. Both parents contributed RSZ increasing alleles. We detected no putative QTL in the joint composite interval mapping for RDR and RSZ.

### LH51×CRW8-1

Six putative QTL for RDR were detected on chromosomes 1, 3, 5, 6, 7, and 8, explaining a total of 50.4% of $\sigma^2_g$. The LOD values ranged from 3.79 to 7.19. All alleles increasing the level of resistance were contributed by resistant parent CRW8-1 except for resistance alleles on chromosomes 3 and 6, which was contributed by inbred LH51. QTL on chromosomes 1, 6, 7, and 8 interacted significantly ($P < 0.01$) with the environment. For RRG, QTL on chromosomes 2 and 9 explained a total of 13.3% of $\sigma^2_g$. The QTL on chromosome 9 showed significant ($P < 0.05$) QTL×environment interactions. Both alleles increasing RRG were contributed by CRW8-1. Three QTL were detected for RSZ on chromosomes 1, 7, and 8 explaining 24% of $\sigma^2_g$. Only CRW8-1 contributed QTL alleles that increased RSZ. The QTL on chromosome 1 did not significantly interact with the testing environments, QTL on chromosomes 6, 7, and 8 showed significant ($P < 0.05$) QTL×environment interactions. A joint composite interval mapping for RDR and RSZ detected two QTL on chromosomes 1 and 8 (Table 5). QTL×trait interactions were significant ($P < 0.05$) for the joint QTL on chromosome 1. A further analysis showed that the QTL for RDR and RSZ in chromosomal bin

**Table 2.** Means of parents LH51 and CRW8-1, and 134 Doubled Haploid Lines derived from their cross and evaluated as topcrosses and estimates of variance components and heritabilities among DHL topcrosses for root damage caused by western corn rootworm (western corn rootworm) feeding, root regrowth, and root size evaluated in four environments

| Statistics | Entries | Traits | | |
|---|---|---|---|---|
| | | Root damage | Root regrowth[a] | Root size[b] |
| | | 0–3 scale | 1–6 scale | 1–6 scale |
| Means | | | | |
| LH51 | 1 | 2.18 ± 0.25[c] | 5.83 ± 0.20 | 5.22 ± 0.18 |
| LH51-TC | 1 | 1.30 ± 0.25 | 5.00 ± 0.19 | 3.10 ± 0.18 |
| CRW8-1 | 1 | -/- | -/- | -/- |
| CRW8-1-TC | 1 | -/- | -/- | -/- |
| DHL-TC | 134 | 1.58 ± 0.30 | 5.15 ± 0.29 | 3.31 ± 0.24 |
| Check hybrids | | | | |
| Bt-Hybrid | | 0.00 ± 0.25 | 5.75 ± 0.21 | 2.28 ± 0.19 |
| Non-Bt Iso hybrid | | 1.01 ± 0.26 | 4.93 ± 0.21 | 3.00 ± 0.19 |
| B37×H84 insecticide | | 0.72 ± 0.25 | 5.14 ± 0.22 | 3.00 ± 0.20 |
| B37×H84 | | 1.72 ± 0.26 | 5.33 ± 0.19 | 3.75 ± 0.18 |
| Variance components (DHL topcrosses) | | | | |
| $\sigma^2_g$ | | 0.0354** | 0.0323** | 0.0129* |
| $\sigma^2_{ge}$ | | 0.0310* | 0.0502** | 0.0247* |
| $\sigma^2_{error}$ | | 0.4727** | 0.4250** | 0.3252** |
| Broad-sense heritability[c] | | 0.43 ± 0.09 | 0.40 ± 0.09 | 0.28 ± 0.12 |

[a]Root regrowth was assessed using a rating scale from 1 (a large amount of regrowth) to 6 (no regrowth).

[b]Root size was assessed using a rating scale from 1 (large root) to 6 (small root).

[c]SEs are attached.

*,**Variance components were significant at the 0.05 or 0.01 probability level, respectively.

**Table 3.** Phenotypic and genotypic (in italics, below diagonal) correlation coefficients among root damage rating, root regrowth, and root size in two mapping populations

| | RDR[a] | RRG | RSZ |
|---|---|---|---|
| Population NGSDCRW1×AG1 | | | |
| RDR | | 0.42** | 0.41** |
| RRG | *0.37*[ns] | | 0.29** |
| RSZ | *0.74*[c] | *0.28*[ns] | |
| Population LH51×CRW8-1 | | | |
| RDR | | 0.32** | 0.59** |
| RRG | *0.48*[c] | | 0.33** |
| RSZ | *0.91*[c] | *0.32*[b] | |

[a]RRG, root regrowth; RDR, root damage rating; RSZ, root size.

[b,c]Genotypic correlation exceeded one or two times its standard error, respectively; ns, nonsignificant.

**Phenotypic correlation coefficient was significant at the 0.01 probability level.

1.07 are most likely two separate QTL in close linkage instead of a single QTL with pleiotropic effects on RDR and RSZ. No QTL×trait interaction was detected for the joint QTL in chromosomal bin 8.06. The hypothesis that both QTL detected in the single trait mapping analysis are one QTL with pleiotropic effects on RDR and RSZ could not be rejected.

## Discussion

To our knowledge, no reports in the refereed literature are available reporting QTL for host plant resistance of maize against western corn rootworm. This does not come to a surprise given the challenges of reliably evaluating maize germplasm for western corn rootworm resistance in highly variable field environments. Our field experiments showed a large genotype × environment interaction and error variances for all resistance traits (Tables 1 and 2). For RDR, the ratio of variance component estimates $\hat{\sigma}^2_g : \hat{\sigma}^2_{ge} : \hat{\sigma}^2_e$ was approximately 1: 2: 13 in both mapping populations resulting in low heritabilities. Even though we developed and tested two segregating mapping populations derived from parental inbreds with contrasting responses to western corn rootworm feeding and already expected a reduced level of genotypic variance expressed among topcrossed doubled haploids, the observed genotypic variances were surprisingly small and the error variances surprisingly high.

Over the last four decades, a diverse set of maize germplasm was evaluated in efforts to identify resistance sources, estimate quantitative genetic parameters for resistance traits, or determine resistance mechanisms. Owens et al. (1974) evaluated 221 inbred lines for western corn rootworm resistance in one location using an experimental design with four replications, and also estimated a low heritability ($\hat{h}^2$ = 0.20). However, heritability estimates for RRG and RSZ were high. Branson et al. (1982) tested a resistant (SD10) and a susceptible (A632) inbred line for RDR, grain yield, and western corn rootworm adult emergence at increasing rates of infestation with western corn rootworm eggs. Even though only the resistant line maintained yield under high infestation levels, the authors were not able to find significant differences between the inbreds for RDR. Branson et al. (1982) speculated that the larger root size of SD10 could explain its level of western corn rootworm tolerance. Evaluating a small set of western corn rootworm resistant and susceptible hybrids, Branson et al. (1983) observed only small, in most cases not significant, differences between resistant and susceptible hybrids for root damage and the number of emerged adult beetles. Interestingly, adults emerged from resistant hybrids were significantly heavier than adults obtained from susceptible hybrids. Riedell and Evenson (1993) tested 11 single cross hybrids from the 1960s, 1970s, and 1980s in a 2-yr study. No significant differences

**Table 4.** Parameters associated with QTL for root damage ratings, root regrowth, and root size

| Bin[a] | Position | LOD | Genetic effect[b] | $R^2_{Partial}$[c] | QTL×E[d] |
|---|---|---|---|---|---|
| | cM | | | | |
| Root damage rating (RDR) | | $T_{0.05}$ = 2.98[e] | 0–3 scale | | |
| 2.02 | 108 | 3.04 | −0.05 | 9.1 | ns |
| 7.02 | 93 | 3.99 | 0.05 | 6.3 | ** |
| 7.06 | 181 | 3.72 | 0.06 | 10.5 | ** |
| 9.04 | 82 | 3.27 | −0.05 | 5.6 | ** |
| 10.03 | 15 | 7.85 | −0.07 | 14.0 | ** |
| | | | $Q^{2f}$ | 29.2 | |
| Root regrowth (RRG) | | $T_{0.05}$ = 2.86 | 1–6 scale | | |
| 1.08 | 226 | 3.41 | −0.06 | 6.2 | ns |
| 2.02 | 93 | 3.17 | −0.06 | 8.5 | ns |
| 6.05 | 2 | 4.73 | −0.08 | 9.7 | ** |
| 8.02 | 14 | 3.21 | −0.05 | 6.0 | ns |
| 9.05 | 117 | 5.74 | −0.05 | 11.4 | ns |
| | | | $Q^2$ | 31.9 | |
| Root size (RSZ) | | $T_{0.05}$ = 3.18 | 1–6 scale | | |
| 1.01 | 18 | 3.64 | 0.05 | 7.4 | ns |
| 1.04 | 110 | 4.93 | −0.05 | 8.2 | ns |
| 10.07 | 80 | 3.54 | 0.09 | 14.4 | * |
| | | | $Q^2$ | 24.2 | |

Parameters were estimated from the phenotypic data of 142 topcrossed DH lines derived from the cross NGSDCRW1×AG1.

[a]BIN locations are designated by an X.Y code, where X is the linkage group containing the BIN and Y is the location of the BIN within the linkage group (Gardiner et al. 1993).

[b]Additive genetic effects were estimated in a simultaneous fit using QTL Cartographer's Multiple Interval Mapping (MIM).

[c] $R^2_{Partial}$ .=.Proportion of phenotypic variance explained by the respective QTL.

[d]*,** QTL×E interactions were significant at the 0.1, 0.05, or 0.01 probability level, respectively; ns = not significant. The significance of QTL×Environment (QTL×E) interactions was tested applying an *F*-test using the test statistic *F* = Mean Square [QTL×E]/Mean Square[Residual×E] (Bohn et al. 1996).

[e]LOD thresholds for genome-wide significance levels of 0.05.

[f]$Q^2$ = Proportion of the genetic variance explained by all detected putative QTL.

for RDR between hybrids were observed. However, hybrids from the 1980s had larger root systems and increased root pulling resistance than hybrids from the 1960s or 1970s. Associated with these improved root characteristics the 1980s hybrids also were more tolerant to western corn rootworm larvae feeding. Finally, Prischmann et al. (2007) evaluated 10 synthetic maize populations in a 2-yr experiment and identified genotypes displaying tolerance as well as significantly reduced root damage and western corn rootworm adult emergence. Unfortunately, Riedell and Evenson (1993) and Prischmann et al. (2007) did not report $\hat{\sigma}^2_g$ or $\hat{h}^2$ values that would allow a more detailed comparison to our study.

A series of factors, inherent to field experiments dealing with maize western corn rootworm resistance might contribute to the large $\hat{\sigma}^2_{ge}$ and $\hat{\sigma}^2_e$ variances. Both mapping populations were evaluated in four environments. These environments varied for soil characteristics, western corn rootworm larvae number, and related feeding pressure, as well as weather conditions. To manage the large genotype × environment interaction and error variances, we selected a subset of environments for each mapping population that maximized $\hat{\sigma}^2_g$ and subsequently heritability estimates. Using this approach, we removed the Urbana environment before conducting a final analysis of population NGSDCRW1×AG1. The trap crop location in Urbana provided extreme insect pressure, which prohibited the detection of small difference between DHL topcrosses for western corn rootworm host plant resistance.

Flint-Garcia et al. (2009) evaluated a series of diverse inbreds and their topcross hybrids for RDR and found no significant relationship between inbred per se and topcross performance. Given that, in corn breeding, the primary goal is to increase topcross or hybrid performance rather than inbred per se performance, we followed the advice of Flint-Garcia (2009) and crossed all DH lines with a susceptible tester. As expected, we observed lower RDR, more RRG, and larger RSZ for topcrosses of the parental inbreds than for the inbreds per se (Tables 1 and 2). Also, the use of vigorous topcrosses has the additional benefit of reducing the genotype × environment interaction and error variances and allowing for a more precise characterization of the DHLs response to western corn rootworm larvae feeding. However, these beneficial effects were offset by the smaller estimates of $\hat{\sigma}^2_g$ for topcrosses than for inbreds (Presterl et al. 2002, Mihaljevic et al. 2005, Flint-Garcia et al. 2009).

Recent reports demonstrate the effect of plant-mediated interactions between leaf and root feeding insects (Erb et al. 2015, Lu et al. 2016, Varsani et al. 2016). Erb et al. (2015) showed that leaf feeding by *Spodoptera littoralis* induced changes in root phenolic acid biosynthesis. Western corn rootworm larvae were able to sense these changes and avoided the infested plant, which resulted in significantly reduced root herbivory. Further studies by Lu et al. (2016) found that this response is conserved across multiple leaves and root feeding insect species as well as corn inbreds. Whether these leaf herbivore-induced changes in root phenolic acid patterns are sufficiently substantial in our germplasm to affect western corn rootworm larvae feeding under field conditions is unknown. Since we did not control for leaf and ear-feeding insects in our experiment this 'herbivore-induced systemic root resistance' could have contributed to the large error variance observed in our study.

The power of a QTL study, i.e., the probability to find putative QTL involved in the inheritance of a plant phenotype, depends on the type and size of the mapping population, density of the genetic

**Table 5.** Parameters associated with QTL for root damage ratings, root regrowth, and root size

| Bin[a] | Position | LOD | Genetic effect[b] | $R^2_{Partial}$[c] | QTL×E[d] |
|---|---|---|---|---|---|
| | cM | | | | |
| Root damage ratings (RDR) | | $T_{0.05}$ = 3.28[e] | 0–3 scale | | |
| 1.07 | 66 | 4.29 | 0.12 | 6.4 | ** |
| 3.05 | 4 | 5.18 | –0.07 | 8.9 | ns |
| 5.03 | 103 | 3.79 | 0.05 | 5.3 | ns |
| 6.01 | 243 | 5.17 | –0.07 | 10.4 | ** |
| 7.02 | 142 | 4.13 | 0.09 | 5.4 | ** |
| 8.06 | 141 | 7.19 | 0.11 | 10.2 | ** |
| | | | $Q^2$[f] | 50.4 | |
| Root regrowth (RRG) | | $T_{0.05}$ = 2.42 | 1–6 scale | | |
| 2.02 | 5 | 2.70 | 7.4 | 8.7 | ns |
| 9.04 | 89 | 2.56 | 8.6 | 4.5 | + |
| | | | $Q^2$ | 13.3 | |
| Root size (RSZ) | | $T_{0.05}$ = 2.87 | 1–6 scale | | |
| 1.07 | 42 | 3.89 | 5.3 | 9.3 | ns |
| 7.04 | 301 | 2.97 | 5.0 | 5.8 | ** |
| 8.06 | 140 | 5.11 | 8.5 | 10.6 | ** |
| | | | $Q^2$ | 24.0 | |
| Multi-trait analysis (RDR, RSZ) | | $T_{0.10}$ = 5.02 | | LOD (QTL×Trait)[g] | |
| 1.07 | 66 | 6.51 | 0.07 | 4.0* | |
| 8.06 | 135 | 5.14 | 0.09 | 0.8 | |

Parameters were estimated from the phenotypic data of 134 topcrossed DH lines derived from the cross LH51×CRW8-1.

[a]BIN locations are designated by an X.Y code, where X is the linkage group containing the BIN and Y is the location of the BIN within the linkage group (Gardiner et al. 1993).

[b]Additive genetic effects were estimated in a simultaneous fit using QTL Cartographer's Multiple Interval Mapping (MIM).

[c] $R^2_{Partial}$ = Proportion of phenotypic variance explained by the respective QTL.

[d]+, *, ** QTL×E interactions were significant at the 0.1, 0.05, or 0.01 probability level, respectively; ns = nonsignificant. The significance of QTL×Environment (QTL×E) interactions was tested applying an *F*-test using the test statistic *F* = Mean Square [QTL×E]/Mean Square[Residual×E] (Bohn et al. 1996).

[e]LOD thresholds for genome-wide significance levels of 0.05.

[f]$Q^2$ = Proportion of the genetic variance explained by all detected putative QTL.

[g]The threshold used for testing QTL×Trait interaction: LOD = 3.2.

linkage map, and the genetic complexity of the trait under study. In contrast to many other QTL studies reported in the literature for insect resistance in maize, our populations were relatively small, and the traits we evaluated showed low but significant heritabilities.

Comparing our results with other published QTL studies is one way to gauge the relevance of our findings. Meihls et al. (2012) summarized the results of 50 published QTL studies conducted for insect resistance in maize. The majority of QTL studies focused on leaf feeding, ear-damaging, and stalk tunneling insect species (e.g., *Ostrinia nubilalis*, *Diatraea grandiosella*, *Diatraea saccharalis*), as well as storage pests (e.g., *Sitophilus zeamais*). These studies used population sizes of 119 to 300 genotypes with a median population size of 178. The number of QTL reported ranged from 1 to 21 QTL per trait with a median of five QTL per trait. Using the summary statistics from this collection of QTL studies (see Table 1 of Meihls et al. 2012), a relationship between population size and QTL number was not apparent. However, given the diversity of these studies concerning the germplasm used, the level of resistance and susceptibility of parental inbreds, and the unknown complexity of the different resistance traits, makes it impossible to draw any sound conclusions regarding the optimum design of a QTL study for insect resistance in maize. Meihls et al. (2012) also projected the QTL to the B73 sequence and found that the vast majority of chromosomal bins (90 out of 100) carried QTL contributing to host plant resistance to above-ground biomass feeding insects. This finding does not surprise given what we know about the diversity of the biochemical pathways and their genetic complexity involved in the host plant resistance response. All QTL we identified for RDR were also located in chromosomal bins already known to carry insect resistance QTL.

In a previous experiment (unpublished data), we subjected seedling roots of a maize hybrid to western corn rootworm larvae feeding, mechanical wounding, and an untreated control treatment to test for differences in metabolic profiles. There were significant differences in the relative abundances of 20 metabolites in western corn rootworm fed versus mechanically wounded roots, but there were only two metabolites that differed in both damage treatments compared with controls. Ascorbate/dehydroascorbate and members of various branches of its biosynthetic pathway including *myo*-inositol, fructose-6-P, glucose-6-P, and glucose were increased in western corn rootworm fed roots compared with control and mechanically wounded roots, implicating ascorbate-mediated defense and hydrogen peroxide signaling in response to western corn rootworm herbivory.

We located QTL for RDR in or adjacent to chromosomal bins known to carry genes/gene models predicted to code for proteins involved in key biochemical reactions of L-ascorbate and β-caryophyllene biosyntheses, and superoxide radicals degradation (Supplementary Information B—Putative Genes, Table S1). Ascorbate has been associated with herbivory performance and host plant response in a variety of ways. Ascorbate is a cofactor for the production of hydroxyproline-rich glycoproteins during cell

wall synthesis (Smirnoff 2000). Arrigoni (1979) demonstrated that ascorbate levels were related to resistance of tomato to the root-knot nematode *Meloidogyne incognita*, and that resistant cultivars exhibited increased ascorbate production after attack, while susceptible cultivars did not. *Spodoptera littoralis* (Boisduval) larval weight gain was increased on an *Arabidopsis* mutant with highly reduced ascorbate levels due to reduced myrosinase activity (Conklin et al. 1997, Schlaeppi et al. 2008). A proteomics study of soybean cyst nematode (SCN) resistance mediated by the multi-genic *rhg1* locus found ascorbate oxidase, dehydroascorbate reductase, and monodehydroascorbate reductase to be constitutively more abundant in resistant plants compared with the susceptible near-isogenic line and led the authors to conclude that constitutively higher expression of the ascorbate pathway may mediate innate SCN resistance (Afzal et al. 2009). Oxidized ascorbate has been implicated as a toxic herbivory defense product via ascorbate oxidase (Felton and Summers 1993). Reduced ascorbate is a fundamental component of hydrogen peroxide scavenging via ascorbate peroxidase, APX (Smirnoff 2000) and lowered amounts of reduced ascorbate have been suggested as a means of plant defense in response to herbivory (Bi and Felton 1995). Suppressed hydrogen peroxide scavenging by APX is a major component of pathogen defense (Smirnoff 2000), and increased hydrogen peroxide has wound sterilization, cell wall strengthening, and herbivory signaling functions (van Breusegem et al. 2001, Orozco-Cárdenas et al. 2001). Ascorbate has essential functions for insects, including protection of the midgut epithelium from oxidative phenolics and tannins and influencing susceptibility to pathogens, and there is evidence herbivorous insects can affect redox status related to ascorbate, as well as its biosynthesis in host plants (Goggin et al. 2010). Studies involving the effect of too much or too little ascorbate in insect diets, including Lepidopterans and Coleopterans, have demonstrated negative health and developmental consequences for both cases (Goggin et al. 2010). In summary, prior research has indicated many ways for ascorbate to contribute to quantitative herbivory resistance in a range of plant species.

Maize plants damaged by western corn rootworm larval root feeding emit the sesquiterpene volatile (*E*)-β-caryophyllene and this compound attracts entomopathogenic nematodes known to parasitize and kill western corn rootworm larvae (Rasmann et al. 2005, Hiltpold and Turlings 2008). The gene *terpene synthase23* (*tps23*) codes for the enzyme (*E*)-β-caryophyllene synthase, which facilitates the dephosphorylation of (2*E*,6*E*)-farnesyl diphosphate to (*E*)-β-caryophyllene. The *tps23* gene was mapped to chromosomal bin 10.03 (Kollner et al. 2008). Whether the QTL for RDR in bin 10.03 is associated with *tps23*, or with a gene of the L-ascorbate biosynthesis pathway, or shows the combined effect of both genes needs to be investigated further. Previous studies suggested that most North American maize germplasm lost the ability to attract nematodes due to the lack of (*E*)-β-caryophyllene (Kollner et al. 2008). Interestingly, the authors were not able to associate the loss of this defensive trait to a DNA sequence mutation of *tps23* or its promotor region. Kollner et al. (2008) speculated that the very low or absent transcript levels of *tps23* in North American maize are caused by the inactivation of a transcription factor or an enhancer element outside of the promotor region. In contrast to resistant parent CRW8-1, resistant parent NGSDCRW1 was partially derived from diverse Pioneer Hi-Bred International germplasm including inbreds from the West Indies and Mexico (Kahler et al. 1985). So, it is possible that the DHLs of the NGSDCRW1×AG1 mapping population segregate for (*E*)-β-caryophyllene production. However, the negative sign of the effect size of the QTL identified in bin 10.03 indicates that AG1 contributed the putative gene underlying this QTL. AG1, a yellow dent line adapted to the US Corn belt, most likely does not express *tps23*. It is interesting to note that Fantaye et al. (2015) increased the susceptibility of maize inbreds to *Colletotrichum graminicola* and *Fusarium graminearum* when restoring (*E*)-β-caryophyllene production.

Eight of the 11 QTL identified for RDR across both mapping populations showed significant QTL × environment interactions. Also, most RDR QTL were population-specific. Only a QTL in chromosomal bin 7.02 was consistently detected in both mapping populations. The complexity of the multiple biochemical pathways involved in western corn rootworm host plant resistance, the large $\hat{\sigma}^2_{ge}$ variances observed for each population, and the diverse sources of germplasm used to develop these populations can explain the lack of consistence of QTL across environments and germplasm. However, it is important to point out that for all three traits

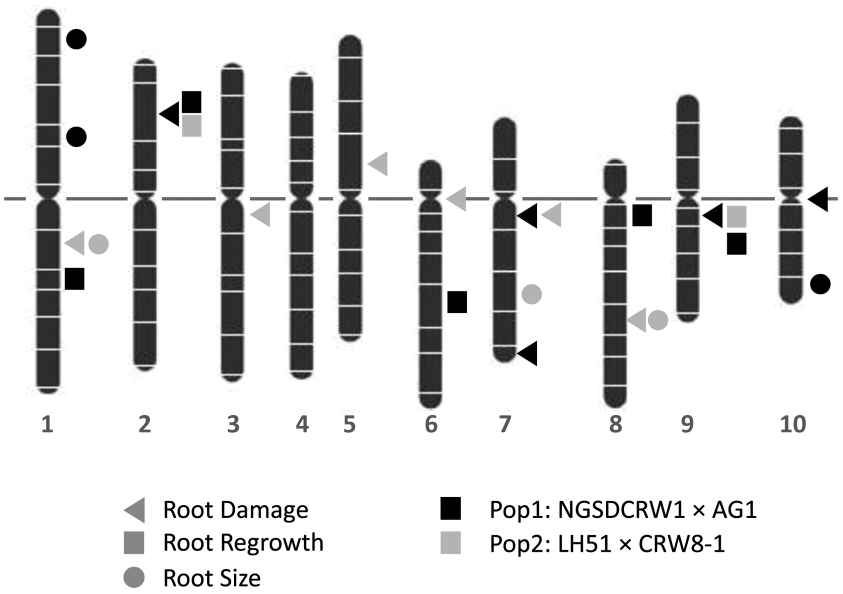


**Fig. 1.** Drawing showing chromosomal BIN position of QTL for root damage, root regrowth, and root size identified in two mapping populations of topcrossed doubled haploids.

associated with western corn rootworm resistance, the majority of QTL alleles increasing resistance were contributed by the resistant inbred line. Inbreds CRW8-1 and NGSDCRW1 were derived from populations improved by recurrent selection focusing on root damage caused by larval feeding, root regrowth, and root size.

The root size and the ability to regrow roots, which were destroyed by larvae feeding, are associated with tolerance of maize against western corn rootworm. For both tolerance traits, we observed significant differences between DHLs in both mapping populations and a positive phenotypic and genotypic relationship between antibiosis and tolerance traits. In general, roots with less root feeding damage were larger and able to outgrow the effects of herbivory damage than roots with high RDR values. However, the correlation coefficients were only of small to moderate size (Table 3). Owens et al. (1974) and Riedell and Evenson (1993) attributed improvement of western corn rootworm host plant resistance mainly to improved tolerance associated with increased roots size and regrowth after damage. Based on our findings, we expect that improving tolerance to western corn rootworm will also improve indirectly the level of antibiosis. However, given the correlation structure among resistance traits observed in our mapping populations, we expect that this effect will be small.

To our knowledge, we also report the first QTL results for RRG and RSZ in maize infested with western corn rootworm larvae. Within and among populations, chromosomal bins 1.07, 2.02, 7.02, 8.06, and 9.04 contain QTL for at least two host plant resistance traits (Fig. 1). For most of these QTL clusters, we do not know whether the genes underlying these QTL belong to trait-specific biochemical pathways or have pleiotropic effects on pathways affecting resistance traits. However, for the RDR and RSZ QTL located in chromosomal bins 1.07 and 8.06 of population LH51 × CRW8-1 we were able to test hypotheses of QTL × trait interaction and pleiotropy versus close linkage. Whereas pleiotropy could not be confirmed for RDR and RSZ QTL in bin 1.07, the hypothesis of pleiotropy could not be rejected for the QTL detected for these traits in bin 8.06. This study provides first insights into the genetic basis of western corn rootworm resistance in maize. However, before this information can be utilized efficiently in breeding programs, additional trials are necessary to confidently determine QTL stability across environments and germplasm and gain insights into the biochemical pathways involved in the plant's response to this important herbivore.

## Supplementary Material

Supplementary data are available at *Journal of Economic Entomology* online.

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