J Chem Ecol (2017) 43:1109–1123
https://doi.org/10.1007/s10886-017-0904-2

A Maize Inbred Exhibits Resistance Against Western Corn Rootworm, *Diabrotica virgifera virgifera*

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Received: 12 April 2017 / Revised: 26 October 2017 / Accepted: 6 November 2017 / Published online: 18 November 2017

Abstract Insect resistance against root herbivores like the western corn rootworm (WCR, *Diabrotica virgifera virgifera*) is not well understood in non-transgenic maize. We studied the responses of two American maize inbreds, Mp708 and Tx601, to WCR infestation using biomechanical, molecular, biochemical analyses, and laser ablation tomography. Previous studies performed on several inbreds indicated that these two maize genotypes differed in resistance to pests including fall armyworm (*Spodoptera frugiperda*) and WCR. Our data confirmed that Mp708 shows resistance against WCR, and demonstrates that the resistance mechanism is based in a multi-trait phenotype that includes increased resistance to cutting in nodal roots, stable root growth during insect infestation, constitutive and induced expression of known herbivore-defense genes, including ribosomal inhibitor protein 2 (*rip2*), terpene synthase 23 (*tps23*) and maize insect resistance cysteine protease-1 (*mir1*), as well high constitutive levels of jasmonic acid and production of *(E)*-β-caryophyllene. In contrast, Tx601 is susceptible to WCR. These findings will facilitate the use of Mp708 as a model to explore the wide variety of mechanisms and traits involved in plant defense responses and resistance to herbivory by insects with several different feeding habits.

Keywords Defenses · *Diabrotica virgifera virgifera* · Jasmonic acid · Maize · Resistance · Roots

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10886-017-0904-2) contains supplementary material, which is available to authorized users.

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Introduction

Due to the difficulty of studying herbivore resistance and tolerance mechanisms in the rhizosphere, far less is known about belowground than aboveground plant defense responses. Nevertheless, characterization of plant resistance against belowground pests is vital. Resistance and tolerance Are two distinct plant strategies to cope with insect pests. Insect-tolerant plants Are able to maintain fitness despite infestation, while resistant plants have structural and chemical traits that allow them to deter and/or negatively impact the insect (Mitchell et al. 2016). Furthermore, there Are two mechanisms of plant resistance to herbivory that include 1] antixenosis, 2] antibiosis (Mitchell et al. 2016; Painter 1951, 1958; Stenberg and Muola 2017). Antixenosis or non-preference mechanisms deter insect feeding and oviposition (Stenberg and Muola 2017), while antibiosis impairs herbivore performance (Painter 1951; Stenberg and Muola 2017). Although it is difficult to distinguish among these mechanisms when studying roots, it is clear that plant resistance to root-feeding pests involves a wide range of defense responses that Are most likely linked to a combination of physical traits, signaling pathways and deterrent biomolecules and the ability

to tolerate herbivory and or regrow after insect attack (Moore and Johnson 2017; Rasmann and Agrawal 2008).

One model system for studying defenses to root herbivory is the important and widely distributed crop, maize (*Zea mays* L.) that is attacked by the western corn rootworm (WCR, *Diabrotica virgifera virgifera*). WCR is a specialist pest of maize in North America (Hummel 2003) that causes economic losses in maize production in North America and Europe (Flagel et al. 2015; Gray et al. 2009; Hummel 2007; Tinsley et al. 2013). It has been estimated that WCR is responsible for more than $1 billion of losses and pest control expenses in maize annually (Gray et al. 2009; Tinsley et al. 2016). For a number of years it has been possible to control this insect with insecticides, crop rotation with soybeans (Levine et al. 2002), and transgenic maize expressing Bt-Cry3Bb1 insecticidal protein (Moellenbeck et al. 2001; Vaughn et al. 2005). Unfortunately, some WCR populations have become resistant to these management strategies (Flagel et al. 2015; Gassmann et al. 2011; Levine et al. 2002; Meinke et al. 1998) and finding additional sources of sustainable resistance is essential. Most of the breeding programs looking for WCR resistance have considered the phenotype of reduced lodging, but this is an indirect way to assess resistance since root antibiosis or toxicity to WCR is not measured. Therefore, this type of screening leads to lodging-tolerant and not necessarily insect-tolerant or insect-resistant lines.

Exploiting innate or native defense tactics of maize is another strategy for identifying sustainable resistance traits that could be incorporated into hybrid maize. One potential defense against WCR is compensatory growth in response to herbivore attack (Prischmann et al. 2007; Qu et al. 2016; Robert et al. 2014, 2015) that involves reallocation of photosynthate between above and belowground organs. Also, other defenses assist in protecting maize from WCR attack such as elevated levels of hydroxamic acids that cause antibiosis (Assabgui et al. 1993, 1995) and accumulation of the sesquiterpene, *(E)*-β-caryophyllene, which attracts entomopathogenic nematodes that Are natural enemies of WCR to maize roots (Kollner et al. 2008; Rasmann et al. 2005). However, *(E)*-β-caryophyllene also is a signal for WCR larvae to aggregate and locate the host plant (Robert et al. 2012a, b) so Its exact role in resistance is difficult to define. Despite research efforts over 60 years, as of 2009 there were no commercial maize hybrids with innate host plant resistance against WCR (El Khishen et al. 2009; Ivezic et al. 2009). Consequently, there is little known about innate host plant resistance traits against WCR in non-transgenic maize inbreds (Abel et al. 2000; Branson and Krysan 1981; Hummel 2003; Rasmann and Agrawal 2008; Sappington et al. 2006).

Maize insect resistance is a variable and multidimensional trait due to crop genetic and phenotypic diversity (Meihls et al. 2012). For example, maize genotypes could be more or less resistant to WCR due to differences in root architecture (Branson et al. 1982), biomechanical strength (Meihls et al. 2012) and biochemical composition (van Dam 2009). In this study we compared responses to WCR infestation in the insect-resistant maize inbred Mp708 and Its insect-susceptible parent, Tx601 (Williams et al. 1985). Mp708 was developed by traditional plant breeding from an Antiguan landrace (Williams et al. 1985, 1987, 1990) and has demonstrated resistance to three distinct feeding guilds of insects. Resistance to the chewing herbivore fall armyworm (*Spodoptera frugiperda*) has been demonstrated in both the laboratory and field (Williams et al. 1985, 1990). Also, bioassays performed among multiple maize inbreds showed that Mp708 is resistant to the root-feeding insect WCR (Gill et al. 2011), and the phloem-sucking insect, corn leaf aphid (*Rhopalosiphum maidis*) (Louis et al. 2015) in the laboratory and greenhouse.

Expression of chitinases, protease inhibitors and β-1,3-glucanases in maize has been correlated with root hebivory and plant defense responses (Lawrence et al. 2012, 2013). Mp708 resistance to fall armyworm and corn leaf aphid has been linked to acumulation of an insecticidal protease, Maize Insect Resistance 1- Cysteine Protease (MIR1-CP) (Louis et al. 2015; Pechan et al. 2000). MIR1-CP is an insecticidal protease that ruptures the caterpillar peritrophic matrix (Mohan et al. 2006) and rapidly accumulates in the whorl when Mp708 is attacked by fall armyworm (Lopez et al. 2007). To date no studies with Mp708 have correlated WCR feeding with insecticidal protein accumulation. In addition, numerous experiments have demonstrated that neither MIR-CP nor Its transcript *mir1* accumulate in Tx601 (Lopez et al. 2007; Pechan et al. 2000). The lack of *mir1* expression in Tx601 is most likely due to a transposable element insertion in the *mir1* promoter of Tx601 (Luthe, unpublished data). Another defensive protein, RIP2 is toxic to fall armyworm and accumulates at the feeding site in Tx601 and Mp708 (Chuang et al. 2014), but Its mode of action is unknown (Chuang et al. 2014). Importantly, no studies to date have determined if MIR1-CP and RIP2 accumulate in maize roots in response to WCR feeding.

In addition to the accumulation of toxic proteins, the aboveground tissues of Mp708 contain elevated levels of jasmonic acid (JA) prior to herbivore attack and therefore it likely that constitutively high JA levels prime Mp708 for subsequent herbivory (Shivaji et al. 2010). The JA-signaling pathway and JA-family of compounds participate in plant defense responses to root herbivory (Ankala et al. 2009; Erb and Glauser 2010; Koo and Howe 2009). Activation of the JA pathway during herbivory through JA-isoleucine conjugate (Chini et al. 2007; Thines et al. 2007) initiates the synthesis of enzymes involved in JA biosynthesis and accumulation of defensive proteins such as MIR1-CP (Ankala et al. 2013), RIP2 (Chuang et al. 2014), chitinases, and protease inhibitors (Ballare 2011; War et al. 2012), along with production of

volatiles such as *(E)*-β-caryophyllene that attracts WCR as well as Its natural enemies (Capra et al. 2015; Rasmann and Agrawal 2008; Rasmann et al. 2005; Robert et al. 2012a).

To better understand defense response traits of maize to root-feeding herbivores, we need to examine traits of insect-resistant maize genotypes. Thus, the main objective of this study was to characterize resistance in Mp708 by comparing Its responses to WCR to the responses of an insect-susceptible genotype, Tx601. Our results suggest that Mp708 is resistant to WCR due to a suite of defensive mechanisms that include strong nodal roots, compensatory root growth despite infestation, high constitutive and inducible levels of JA, *mir1-cp*, *rip2* and the presence of *(E)*-β-caryophyllene. We propose that Mp708 is a unique maize genotype that is well-suited to be model for investigating herbivore defense responses due to Its resistance to insects with different feeding behaviors.

Methods and Materials

Plants and Insects Seeds for the maize genotypes, insect-resistant Mp708 and Its susceptible parent, Tx601 (Williams et al. 1985) were provided by Dr. Paul Williams, USDA-ARS, Mississippi State University. Plants were grown in Hagerstown Loam in the Plant Science greenhouse at the Pennsylvania State University in 8 cm × 9 cm pots. Supplemental lightening was used to maintain 14 h day and 8 h night cycle and temperature was kept between 22 and 27 °C. Diapause WCR eggs were obtained from Dr. Bryan French, USDA-ARS, Brookings, SD and reared for 10 to 12 d on damp paper towels at 25 °C in the dark until hatching.

Insect Infestation and Tissue Collection Time course experiments were done by infesting each Mp708 and Tx601 plant at the V3 stage (Ritchie et al. 1998) for 2, 4, or 7 d with ~20 WCR, that have hatched within ~24 h. Control plants were not infested with insects (including the 0 d time point). Roots were cleaned, collected, weighed (0.1 g per biological replicate) and stored at −80 °C until further use. In both gene expression and immunoblot experiments a minimum of three biological replicates per treatment were used; a biological replicate was root tips (up to 2 cm from the tip) pooled from two plants.

Root Length and Anatomy Mp708 and Tx601 roots were examined to determine total length and anatomical changes after WCR infestation. After 3, 6 and 9 d of continuous WCR infestation, 10 root systems of control and infested plants were collected, cleaned and stored in 75% (*v*/v) ethanol for further analysis. Root length was measured by separating all the roots from the base of the mesocotyl and scanning them in a flatbed scanner at a resolution of 400 dots per inch (Epson Expression 1680, Seiko Epson Corporation, Suwa, Japan) (Supporting Information Fig. S1). The scanned images were analyzed using WinRhizo software (Arsenault et al. 1995) to determine total root length and the root system was classified into nodal and seminal roots depending on root diameter (Supporting Information Fig. S1). Seminal roots had a diameter between 0.5–1 mm and nodal roots between 1 and 6 mm (Arsenault et al. 1995; Burton et al. 2012). Statistical analysis was performed on the data collected from the WinRhizo software using R statistical software version 3.2.1 (Team RC 2015).

To further characterize differences in injury from WCR on Mp708 and Tx601, we used laser ablation tomography (LAT) to determine the regions of the nodal roots that were attacked by WCR after 9 d of infestation (Chimungu et al. 2014). For LAT, a pulsed laser beam (Avia 7000, 355 nm pulsed laser) was used to ablate root tissue in a camera focal plane as the root segment is advanced with an imaging stage. The cross-section images were taken using a Canon T3i 399 (Canon Inc. Tokyo, Japan) camera with 5X micro lens (MP-E 65 mm). The images were analyzed using ImageJ software (Schneider et al. 2012) and the amount of cortex lost was determined as the percentage of tissue missing from the estimated undamaged area. A minimum of five biological replicates were used per treatment, and one LAT image was used per plant. Root length and percentage of cortex lost were analyzed by using logarithmic, square root, inverse power or box-cox transformations until the Shapiro-Wilk test (Shapiro and Wilk 1965) confirmed normal distribution, then a multiple-factor analysis of variance (ANOVA) was performed for each day individually, followed by a significant difference (HSD) Tukey pairwise comparison test in R version 3.2.1.

WCR Bioassays Bioassays were done by using a small brush to place 20 WCR (~24 h hatched) neonates in the root system, where they were allowed to feed for 4 d. At the end of day 4, the seedlings were individually placed in plastic funnels connected to a vial with 75% (*v*/v) ethanol (Supplemental Information Fig. S2). The funnels were placed at room temperature under a constant light source that dried the soil for 7 d after which the number of WCR recovered was determined and percent survival calculated and analyzed by ANOVA in R version 3.2.1.

Biomechanical Analysis of Nodal Roots To measure the ability of Tx601 and Mp708 roots to resist cutting, a mechanical injury similar to WCR herbivory was simulated, and the maximum cutting stress (mCF) that the nodal roots could withstand was measured. Cutting tests were performed on nodal roots as these roots Are typically targeted by WCR larvae (Hummel 2007; Kadlicko et al. 2010; Oleson et al. 2005). Maize seeds were surface sterilized with 2% (*v*/v) hypochlorite solution and pre-germinated at 20 °C for 48 h prior to planting. Plants were grown in a controlled environment

with a 10 and 14 h night and day cycle, at 20 °C or 24 °C respectively, within plastic tubes 300 mm in height and 150 mm diameter. Within each core, 5.5 L of a soil: sand: vermiculite mix (50:25:25 respectively) was packed to a depth of 280 mm. Prior to planting, the cores were saturated with 850 ml of nutrient solution as described in Zhu et al. (2010). Four replicates of Tx601 and Mp708 were harvested 3 wk. after planting when plants reached the V3 developmental stage (Ritchie et al. 1998). Planting was staggered to ensure sufficient time for biomechanical testing at the V3 stage. Each replicate was tested within 48 h of roots being washed from the growth media eliminating potential confounding errors associated with decomposition. Biomechanical testing was performed using a singled edged razor blade (Ang et al. 2008) with root samples installed over a slotted plastic block and secured in place using elastic bands. Cutting force was recorded using an Instron 5966 universal test frame with a 10 N load cell accurate to ±2.5 g at maximum load. The cutting force was measured during the extension (cutting) phase. Extension was at a rate of 2 mm min^{-1} with maximum cutting force calculated as peak load $root^{-1}$ cross sectional area. For each 60 mm long segment of root multiple cut tests were performed along the axis with a minimum 10 mm between each cut to minimize the risk of influence on the next. Due to root diameter being smaller than the length of the razor blade the following cut test was performed using an unused portion of the blade by moving the root segment. Effectively a new area of the razor blade was used for each test ensuring potential blade blunting did not affect results.

RNA Extraction, and Quantitative Real-Time PCR Analysis Leaf and root tips (up to 2 cm from the root tip) from all root types were ground using a ball-mill tissue grinder (Genogrinder 2000; SpexCentriprep Inc., Metuchen, NJ, U.S.A.) for 2 min at 2,000 strokes min^{-1} under liquid nitrogen conditions. RNA was extracted from all ground tissues using TRIzol®-chloroform protocol, and treated with DNase (New England Biosciences) following manufacturer's instruction. RNA content was measured using a Nanodrop (Thermo Scientific) and cDNA was made using High Capacity cDNA Reverse Transcription Kit (ABI, Foster City, CA) following manufacturer instructions. Real-time PCR analyses were done for time course expression experiments by using primers for *aos, opr7, mpi*, *fpps3, tps23, mir1* and *rip2* and *actin* as endogenous control gene *aos* F: 5′-CAA ACC GAC GAA TTT GAG CA-3′, R: 5′-GGA GGC TCG CAA CAA GTT G -3′; *opr7* F: 5′-CCC ATG GCT ACC TCA TCG AT-3′, R: 5′-CGT CAG TCC GGT CGT TGA T-3′; *rip2* F: 5′-GAG ATC CCC GAC ATG AAG GA-3′, R: 5′-CTG CGC TGC TGC GTT TT-3′; *mpi* F: 5′-GCG GAT TAT CGC CCT AAC C-3′, R: 5′-CGT CTG GGC GAC GAT GTC-3′; *fpps3* F: 5′-CCT GGC TAG TTG TGC AAG CT-3′, R: 5′-GAA AAC AGT TTG GAC TGC CT-3′; *tps23* F: 5′-TCA CCC ATG AGT GCC TCA GA-3′, R: 5′-GTT GAC CGC CCT CTC TAG AAG A-3′; *mir1* F: 5′- GAG GGT CTT GTC GTG TTG AAC TT-3′, R: 5′- GCC ACA CCA TAA CGG ATT AAC TT-3′; *actin* F: 5′-GGA GCT CGA GAA TGC CAA GAG CAG-3′, R: 5′- GAC CTC AGG GCA TCT GAA CCT CTC-3′ The primers were designed using Primer Express software for real-time PCR (version 3.0) (ABI, Foster City, CA). The PCR conditions used were: step 1: 50 °C for 2 min and 95 °C for 10 min, step 2: 95 °C for 15 s and 60 °C for 1 min for 40 cycles, Step 3: 72 °C for 10 min, Step 4: dissociation stage. The relative quantification values were obtained by using ABI 7500 Fast SDS Software (version 1.4) (ABI, Foster City, CA), and analyzed with the R statistical software (Team RC 2015). The data was analyzed by first using logarithmic, square root, inverse, power of two or box-cox transformations until the Shapiro-Wilk test (Shapiro and Wilk 1965) confirmed normal distribution, then a multiple-factor analysis of variances (ANOVA) was done, followed by a significant difference (HSD) Tukey pairwise comparison test in R version 3.2.1.

Jasmonic Acid (JA) Quantification Root tissues were collected as previously described and placed in 2 ml screw-cap FastPrep tubes (Qbiogene, Carlsbad, CA) containing Zirmil beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ). Dihydro-jasmonic acid (dhJA) was added to each vial as internal standard (100 ng) followed by 400 μl of 1-propanol:water:hydrochloric acid (2:1:0.002, *v*/v) and shaken for 40 s in a FastPrep FP 120 tissue homogenizer. Dichloromethane (1 ml) was added to each sample, followed by shaking for 40 s in the homogenizer, and centrifugation at 13,000 x g for 1 min. The bottom dichloromethane and 1-propanol layer was then transferred to a 4 ml glass screw-cap vial and dried under an air stream. Samples were reconstituted in methanol:diethyl ether solution (1:9, *v*/v) and 2.3 μl of trimethylsilyldiazomethane hexane (Aldrich) were added to each. The vials were then capped and allowed to sit at room temperature for 25 min. Excess trimethylsilyldiazomethane was destroyed by adding 2.3 μl of 2.0 M acetic acid in hexane to each sample (Schmelz et al. 2003, 2004). Finally, the phytohormones were collected by using a vapor phase extractions protocol previously described by Schmelz et al. (2004). The extracts were run in a gas chromatograph mass spectrometer with electron ionization and identity and quantity of the total JA was determined by comparing the retention times and spectra of the internal standard. The data was analyzed by using logarithmic, square root, inverse, power of two or box-cox transformations until the Shapiro-Wilk test (Shapiro and Wilk 1965) confirmed normal distribution, then a multiple-factor analysis of variances (ANOVA) was done, followed by a significant difference (HSD) Tukey pairwise comparison test in R version 3.2.1.

Results

Survival of WCR on Tx601 and Mp708 We evaluated the performance of WCR on Tx601 and Mp708 by assessing survival following 4 d of feeding. A significantly higher percentage (35%) of WCR survived when the insects fed upon Tx601 compared to Mp708 (22%, $P < 0.05$) (Fig. 1). This finding confirms previous research (Gill et al. 2011) and supports our use of Mp708 as a model to investigate physiological and biochemical resistance traits that differ from those of Tx601.

Changes in Root Length and Growth during WCR Infestations To characterize the root damage caused by WCR feeding, we measured total root length at 3, 6 and 9 d following WCR infestation (Supplemental Information Fig. S1). Mp708 and Tx601 total root lengths were not significantly different in the controls, but when plants were infested with WCR, Tx601 showed 30–40% less total root length than Mp708 indicating significant differences in genotype x treatment interaction (Fig. 2a, $P<0.05$). Length of nodal roots showed no significant differences between genotypes ($P = 0.281$) or between infested and control plants ($P = 0.166$). Mp708 had longer lateral roots than Tx601 in control plants at 3 d and infested treatments throughout the 9 d period (Fig. 2b, $P < 0.05$). To determine if changes in total root length could be caused by WCR-induced reduction in root growth, we related root length changes with roothairless 3 (*rth3*) gene expression (Hochholdinger et al. 2008) during WCR infestation. This gene is a marker of growth in the root apical meristem (Bassani et al. 2004; Hochholdinger et al. 2008; Rost and Bryant 1996) that encodes a putative GPI-anchored, monocot-specific, COBRA-like protein that has been linked to root hair elongation, various types of cell expansion and cell wall biosynthesis in maize (Hochholdinger et al. 2008). Throughout 7 d of continuous WCR exposure, constitutive expression of *rth3* remained unchanged in Mp708 while it significantly decreased by day 7 in Tx601 (Fig. 2c, $P < 0.05$). Our results suggest that Mp708 maintained root growth in spite of WCR feeding.

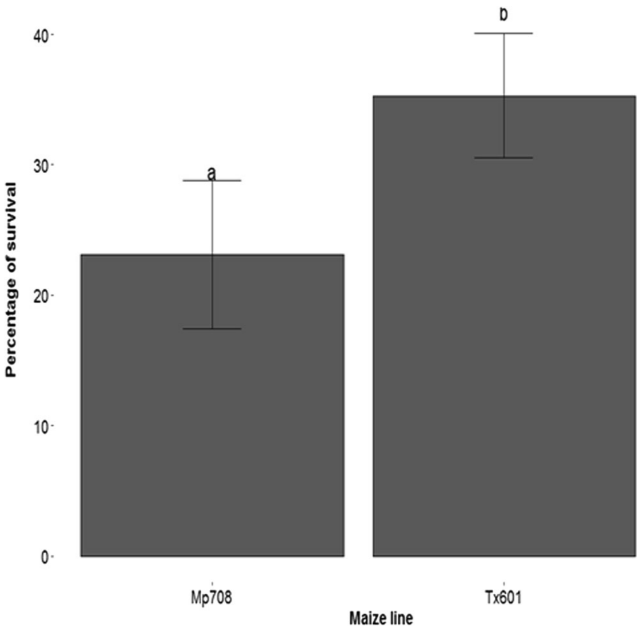

Fig. 1 Percent survival of WCR fed on Mp708 and Tx601 maize lines. Percent survival was normalized and analyzed using multiple-factor ANOVA followed by honest significant difference (HSD) Tukey pairwise comparison test, the results of the HSD Are represented by the letters and the error bars represent the standard error. $n = 14$ for Mp708 and $n = 15$ for Tx601

Differences in Root Anatomy and Strength between Tx601 and Mp708 Root anatomy images captured with the laser ablation technique showed undamaged and damaged tissues resulting from 9 days of WCR infestation (Fig. 3a–d). In general, nodal roots of Mp708 appeared less damaged than those of Tx601 (Supplemental Information Fig. S1). Image analysis of damaged roots showed that WCR typically fed on the root cortex with a 50% higher cortex loss in Tx601 compared to Mp708 (Fig. 3e, $P < 0.05$). By 9 days there were no significant differences in the percentage of stele lost between control and infested tissues ($P = 0.127$).

To assess if there were differences in the nodal root toughness that could explain the higher losses of cortex in Tx601, we used a single edge razor blade system (Ang et al. 2008) and measured the maximum cutting force (mCF) needed to sever nodal roots at various positions from the root tip to the base of the stem. Roots grow acropetally (from the root tip) allowing distance from the root tip to be used as a proxy for root age with tissue age increasing closer to the root base (Loades et al. 2015). Significant differences in mCF were observed between Mp708 and Tx601 ($P < 0.001$) with mCF increasing linearly with increasing root age in Mp708 ($R^2 = 0.673$) (Fig. 3f). In Tx601 there was not a strong correlation between mCF and root age ($R^2 = 0.095$)(Fig. 3f). In Tx601, mCF increased linearly with increasing distance from the root tip up to ~60 mm from the root tip, but beyond this point the mCF was not observed to either increase or decrease indicating a threshold (Fig. 3f).

Expression of two JA Biosynthetic Pathway Genes and JA Accumulation in Roots during WCR Infestation Because herbivory by chewing insects activates JA biosynthesis (Koo and Howe 2009; McConn et al. 1997), the transcript levels of two genes in this hormonal biosynthetic pathway, allene oxidase synthase (*aos*) and oxo-phytodienoate reductase 7 (*opr7*) were measured in WCR-infested roots using RT-qPCR (Koo and Howe 2009; McConn et al. 1997; Yan et al. 2012). Constitutive (day 0) levels of both *aos* and *opr7* transcripts were significantly higher in Mp708 than Tx601. During WCR infestation, *aos* transcripts in Mp708 accumulated

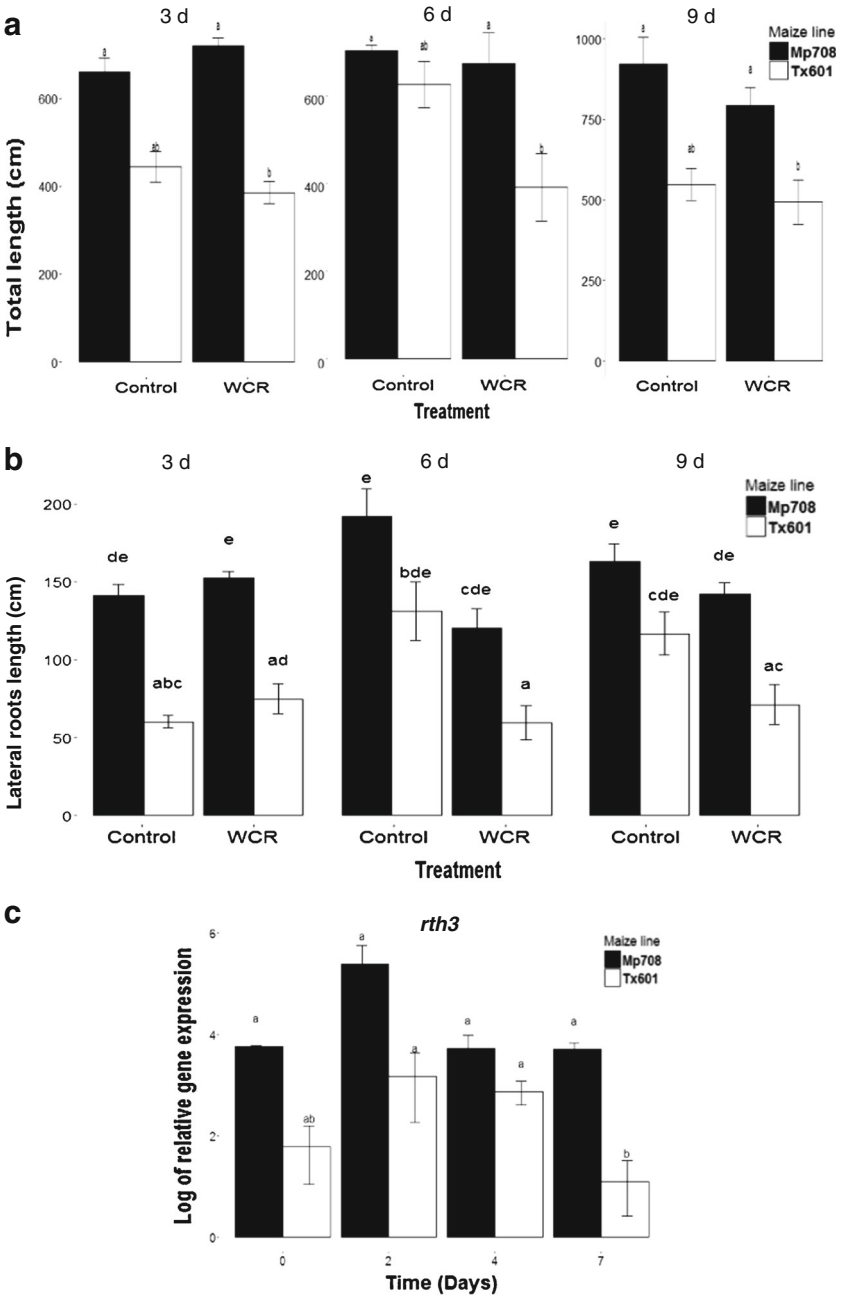

Fig. 2 Total root length in Mp708 and Tx601 at days 3, 6 and 9 after continuous infestation with WCR (**a**). Lateral root length in Mp708 and Tx601 at days 3, 6 and 9 after continuous infestation with WCR (**b**). Time course analysis of *rth3* transcript accumulation in Mp708 and Tx601 in response to continuous infestation with WCR (**c**). Relative expression (RQ) of *rth3* was measured by qRT-PCR. Gene expression levels were normalized to *actin*. Length and RQ data were normalized and analyzed using analyzed using multiple-factor ANOVA followed by honest significant difference (HSD) Tukey pairwise comparison test; $n = 3$ per day for each genotype for RQ data. Letters represent results of the HSD ($P < 0.05$) and error bars show the standard error. For the root analysis, on day 3, $n = 5$ and n = 3 for Mp708 and Tx601 control and n = 5 and $n = 4$ for Mp708 and Tx601 infested; on day 6, n = 3 and n = 4 for Mp708 and Tx601 control and n = 5 and n = 4 for Mp708 and Tx601 infested; on day 9, $n = 7$ and $n = 6$ for Mp708 and Tx601 control and n = 5 and n = 6 for Mp708 and Tx601 infested

significantly and peaked by day 4, while transcript levels in Tx601 remained low and did not change during this time (Fig. 4a; $P < 0.05$). In Mp708, constitutive levels of *opr7* transcripts were significantly higher than in Tx601 and these levels remained high throughout the 7 d infestation. In Tx601, *opr7* transcript abundance gradually increased during the infestation, but transcript levels were only significantly higher than the control on day 7 (Fig. 4b; $P < 0.05$). The results suggest that Mp708 has the capacity to increase production of JA earlier than Tx601 ultimately leading to higher constitutive JA levels in this genotype.

Previous research has shown that Mp708 whorls had higher constitutive JA levels than Tx601 and that these levels increased in response to fall armyworm feeding (Shivaji et al. 2010), which suggested that Mp708 was genetically "primed" to respond to herbivory (Shivaji et al. 2010). To determine if roots showed similar responses, we measured JA in roots of Mp708 and Tx601 under non-infested conditions. We found that Mp708 roots had an approximately 3-fold higher JA concentration than Tx601 prior to WCR-feeding (Fig. 5a, $P < 0.05$). In addition, JA levels in Mp708 roots increased approximately 3-fold in response to WCR feeding after 4 d of infestation (Fig. 5b; $P < 0.05$), which corresponded with the higher levels of *aos* expression in Mp708 (Fig. 4a). Furthermore, JA levels in leaves did not increase in response to belowground WCR infestation (Fig. 5b).

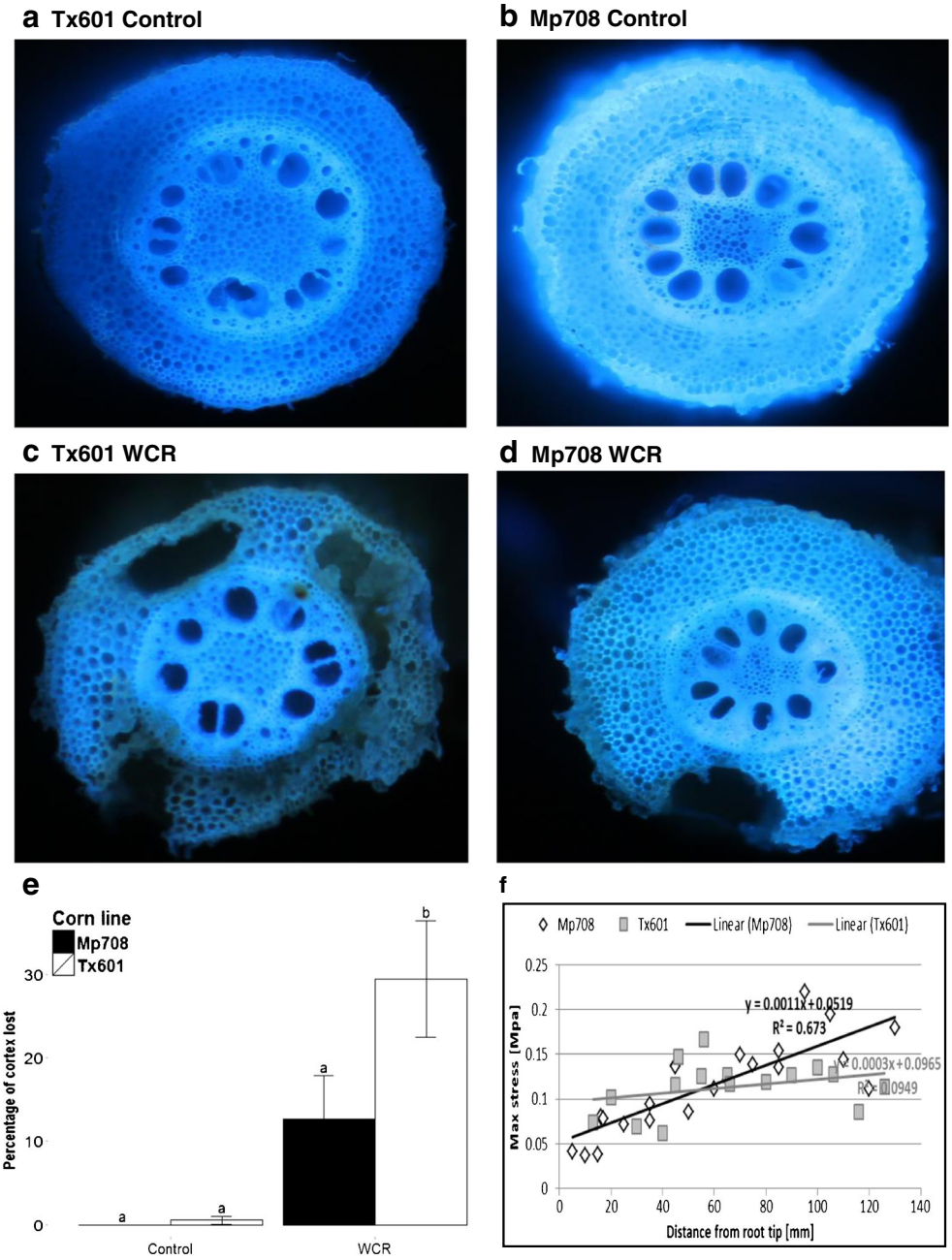

Fig. 3 Laser ablation tomography (LAT) cross-sections from nodal roots of Tx601 and Mp708 after 9 d of continuous infestation with WCR. Root cross-sections from control Tx601 (**a**) or Mp708 (**b**) and WCR-infested Tx601 (**c**) and Mp708 (**d**). Percentage of cortex lost in Mp708 and Tx601 in non-infested control plants or those infested with WCR for 9 days (**e**). Maximum cutting strength in nodal roots (**f**). Linear regression analysis shows a significant difference between lines ($P<0.001$) as a function of distance from stem base. For the LAT images (**e**) the percentage loss was determined from images analyzed with ImageJ software, normalized and analyzed using multiple-factor ANOVA followed by honest significant difference (HSD) Tukey pairwise comparison test. Letters represent results of the HSD ($P<0.05$) and error bars show the standard error; n=6 for Mp708 and Tx601 control, n=5 for Mp708-WCR and n=6 for Tx601-WCR

Accumulation of Defense Genes in Roots in Response to WCR Infestation To better understand the downstream molecular differences between Tx601 and Mp708 caused by WCR feeding, we measured, with RT-qPCR, root transcript levels of five insect-defense related genes: *rip2* (Chuang et al. 2014), maize proteinase inhibitor (*mpi*) (Tamayo et al. 2000; Vila et al. 2005), farnesyl diphosphate synthase 3 (*fpps3*) (Richter et al. 2015), terpene synthase 23 (*tps23*) (Degenhardt et al. 2009; Rasmann et al. 2005; Rasmann and Turlings 2007), and *mir1*.

When infested with WCR, *rip2* expression in Mp708 roots significantly increased and peaked only at day 4, and Its transcript levels were significantly higher than those of Tx601 at days 4 and 7. The expression of *mpi* in Mp708 and Tx601 increased dramatically after 2 days of infestation and remained high in both genotypes (Fig. 6b; $P < 0.05$). These results indicated that *rip2,* but not *mpi,* transcripts accumulate faster and to higher levels in Mp708 than in Tx601 (Fig. 6a, b) and suggest that there Are differences in the expression of direct defense genes between the insect-resistant and susceptible genotypes.

Since terpene-derived compounds appear to be involved in plant defenses (Richter et al. 2015), we measured expression of *fpps3*, which encodes the enzyme involved in producing farnesyl diphosphate (FPP), a precursor of sesquiterpenes,

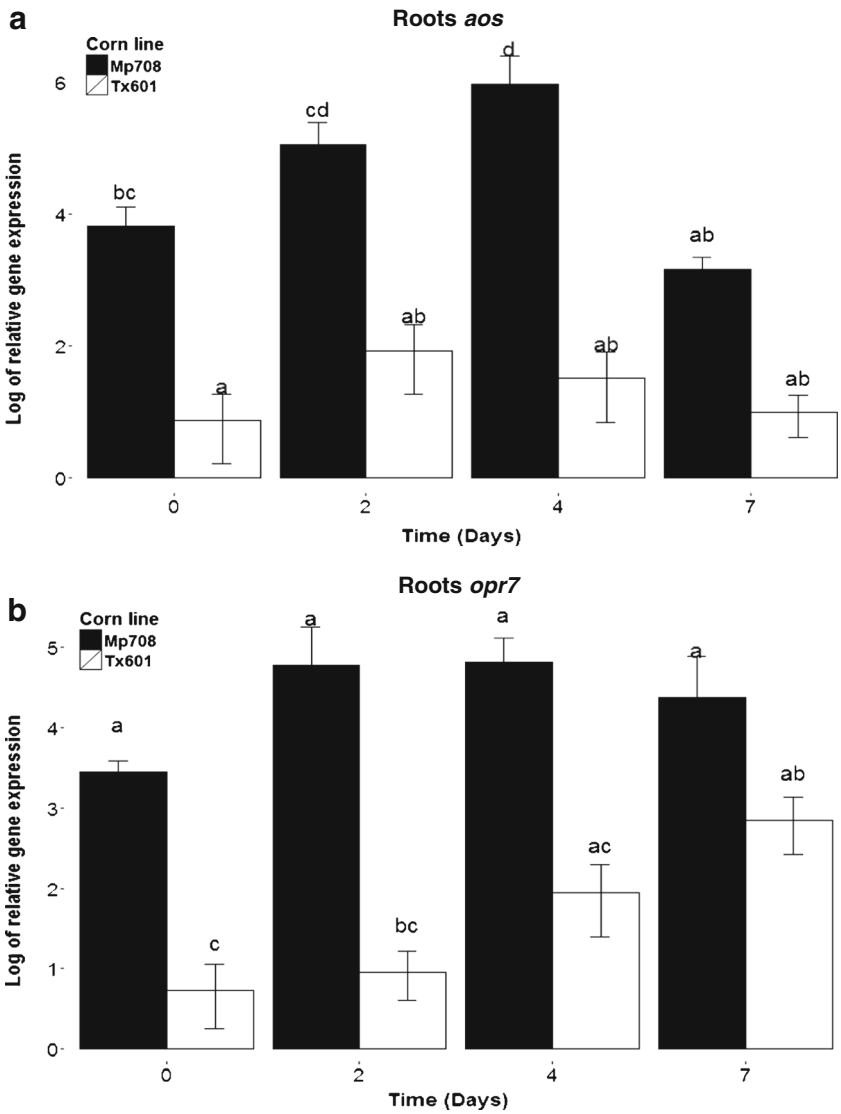

Fig. 4 Time course analysis of jasmonic acid biosynthetic genes of maize in roots of Mp708 and Tx601 in response to WCR infestation. **a** *aos* and **b** *opr7* transcript accumulation. Gene expression levels were determined in V3 stage plants 0, 2, 4 and 7 d after belowground infestation with WCR. Relative expression (RQ) of *aos* and *opr7* was measured by qRT-PCR. Gene expression levels were normalized to *actin*. RQ data were normalized and analyzed using multiple-factor ANOVA followed by honest significant difference (HSD) Tukey pairwise comparison test. Letters represent results of the HSD ($P < 0.05$) and error bars show the standard error; n = 3 per time point and per genotype

polyphenols, squalene, triterpenes and ubiquinones (Richter et al. 2015; Sallaud et al. 2009). We also measured transcript levels of *tps23,* which functions down stream of *fpps3* and facilitates production of the sesquiterepene, *(E)*-β-caryophyllene (Kollner et al. 2008). *fpps3* transcripts significantly increased by day 4 in Mp708 and Tx601, but there were no significant differences between the genotypes (Fig. 7a). In contrast, abundance of *tps23* transcripts in Mp708 increased during WCR infestation and was significantly higher than in Tx601 at all time points. In Tx601, *tps23* transcript levels were low and did not increase during infestation (Fig. 7b). These results suggest that Mp708 is capable of producing *(E)*-β-caryophyllene that could indirectly contribute to WCR resistance by attracting Its natural enemies. In fact, a previous study (Smith et al. 2012) demonstrated that Mp708 plants constitutively produced 10-fold greater levels of *(E)*-β-caryophyllene than Tx601.Futhermore, there were no significant differences in the constitutive and induced *(E)*-β-caryophyllene levels in Mp708. This could send a "decoy" signal indicating that the plant is already infested with WCR and attract entomopathogenic nematodes that Are natural enemies of WCR (Robert et al. 2012b).

We also examined the transcript profile of *mir1,* which is expressed in whorls of Mp708 but not Tx601 (Mohan et al. 2008; Pechan et al. 2000). *mir1* transcript levels increased dramatically in Mp708 roots during WCR infestation and peaked at day 4 (Fig. 8a). Importantly, the *mir1* transcript accumulation coincided with *aos, opr7* expression and JA accumulation profiles (Fig. 5b), suggesting that MIR1-CP insecticidal properties could be contribute to WCR resistance in Mp708 in addition to high constitutive and inducible JA levels, *rip2* and *tps23* expression and the presence of *(E)*-β-caryophyllene.

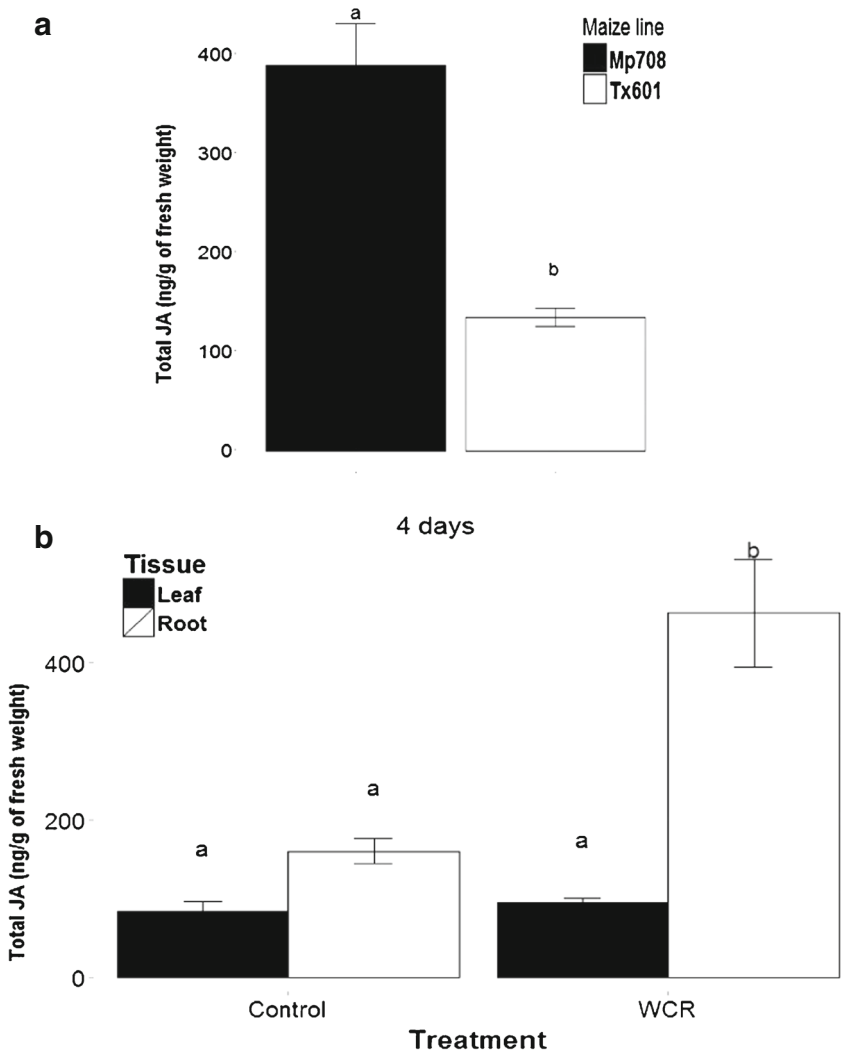

Fig. 5 Analysis of constitutive and induced jasmonic acid (JA) in response to WCR infestation. JA levels in root from (**a**) Mp708 and Tx601 and (**b**) JA accumulation in roots tips and leaves of Mp708 infested with WCR. Control plants were not infested with WCR. JA levels were determined as described in Materials and Methods. JA data were normalized and analyzed using multiple-factor ANOVA followed by honest significant difference (HSD) Tukey pairwise comparison test. Letters represent results of the HSD ($P < 0.05$) and error bars show the standard error; n = 5 for Tx601 and Mp708 and n = 5 for root and leaf tissues per treatment

Discussion

We present data suggesting that Mp708 has resistant traits against WCR that could contribute to antixenosis and antibiosis (Painter 1951, 1958). The traits evaluated include longer root system and stable root growth (Fig. 2), root biomechanical resistance to cutting (Fig. 3), high constitutive and induced JA levels in roots (Fig. 5), synthesis of insecticidal proteins transcript such as *mir1* (Fig. 8) and *rip2* (Fig. 6a) and *(E)*-β-caryophyllene production (Fig. 7). To the best of our knowledge, characterization of innate insect resistance to WCR in non-transgenic maize inbred lines has not been previously reported. Bioassays showed that fewer WCR larvae survived when fed Mp708 roots compared to Tx601 (Fig. 1), validating previously published data from multiple maize lines at the V8 developmental stage that showed fewer larvae recovered from Mp708 plants compared to Tx601 and B73 lines (Gill et al. 2011). Because the current study focused on characterizing traits that contribute to resistance due to antixenosis and antibiosis, we suggest that Mp708 has desirable traits that could be exploited in plant breeding programs targeting resistance to WCR and possibly other root herbivores (Jogaiah et al. 2012).

The results show that Mp708 and Tx601 roots were differentially damaged by WCR feeding. Mp708 lateral roots were longer than those of Tx601 at early developmental stages (3 d) (Fig. 2b) and both Mp708 and Tx601 roots tended to increase in length over time (Fig. 2), but following WCR infestation, the total root length of Tx601 was lower than that of Mp708 because fewer lateral roots were measured in Tx601 (Fig. 2a). Mp708 nodal and lateral roots were more resistant to cutting (Fig. 3f), which could be one reason for lower WCR feeding and survival on Mp708 (Supporting Information Fig. S2). Also, laser ablation images showed that WCR tended to feed on the nodal root cortex and caused more damage in Tx601 than Mp708. These results imply that Tx601 lateral roots Are more prone to damage by organisms with piercing or chewing feeding strategies. The tougher nodal and lateral roots of Mp708 could make it more

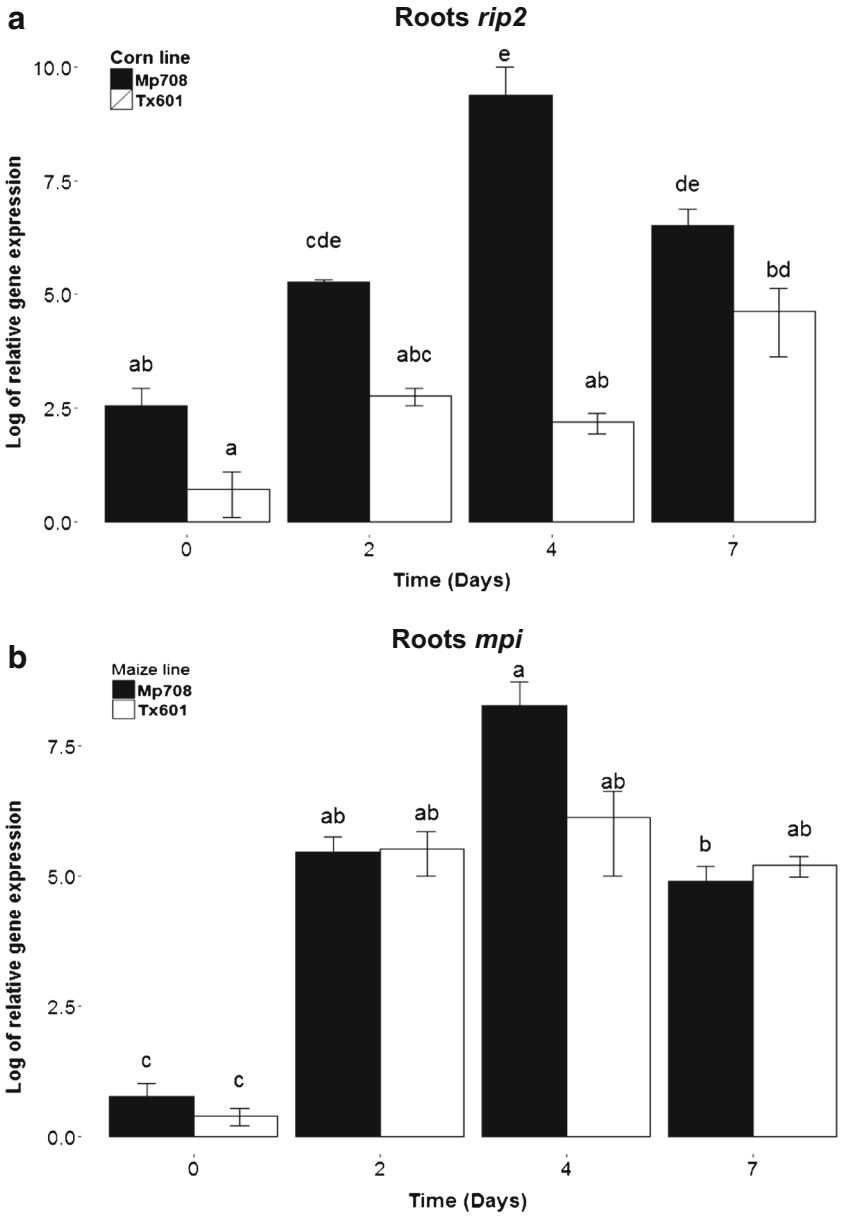

Fig. 6 Time course of maize defense genes in response to WCR. **a** *rip2* and **b** *mpi* transcript accumulation in roots of Mp708 and Tx601. Gene expression levels were determined in V3 stage plants 0, 2, 4 and 7 d after belowground infestation with WCR. Relative expression (RQ) of *rip2* and *mpi* were measured by qRT-PCR. Gene expression levels were normalized to *actin*. RQ data were normalized and analyzed using multiple-factor ANOVA followed by honest significant difference (HSD) Tukey pairwise comparison test. Letters represent results of the HSD ($P < 0.05$) and error bars show the standard error; n = 3 per time point and per genotype

difficult for WCR to feed on the roots and access nutrients. In tobacco, decreased root toughness and lignin accumulation has been linked to low tolerance to root wireworms due to weaker root tension revealed by a fracture toughness test (Johnson et al. 2010). That study only observed significant differences in fracture toughness between tobacco lines, not in resistance to resistance (Johnson et al. 2010).

Furthermore, loss of lateral root length could contribute to the poorer performance of Tx601 compared to Mp708 plants under WCR infestation, since lateral roots Are responsible for most of nutrient and water uptake contributing to overall plant fitness (Paez-Garcia et al. 2015). In Tx601, the reduction in root length could be the result of both greater root consumption by WCR and reduced root growth. It is possible that WCR is feeding on lateral roots, or that feeding on nodal roots affects development of lateral roots via either regulatory mechanisms or resource limitation. The mechanisms that cause root growth differences between Mp708 and Tx601 remain unclear.

Expression profiles showed that Mp708 was able to maintain *rth3* transcript levels during infestation, whereas in Tx601 *rth3* expression decreased significantly by day 7 (Fig. 2c). Maintenance of root growth during WCR infestation could result in similar shoot biomass and CO_2 assimilation as uninfested plants (Riedell and Reese 1999), leading to unaffected yields (Branson et al. 1982). Taken together, these results suggest that Mp708 has a root system that is more resistant to WCR feeding than the root system of Tx601 so that Mp708 is able to maintain root growth during infestation. These findings combined with the production of potentially toxic proteins like MIR1-CP

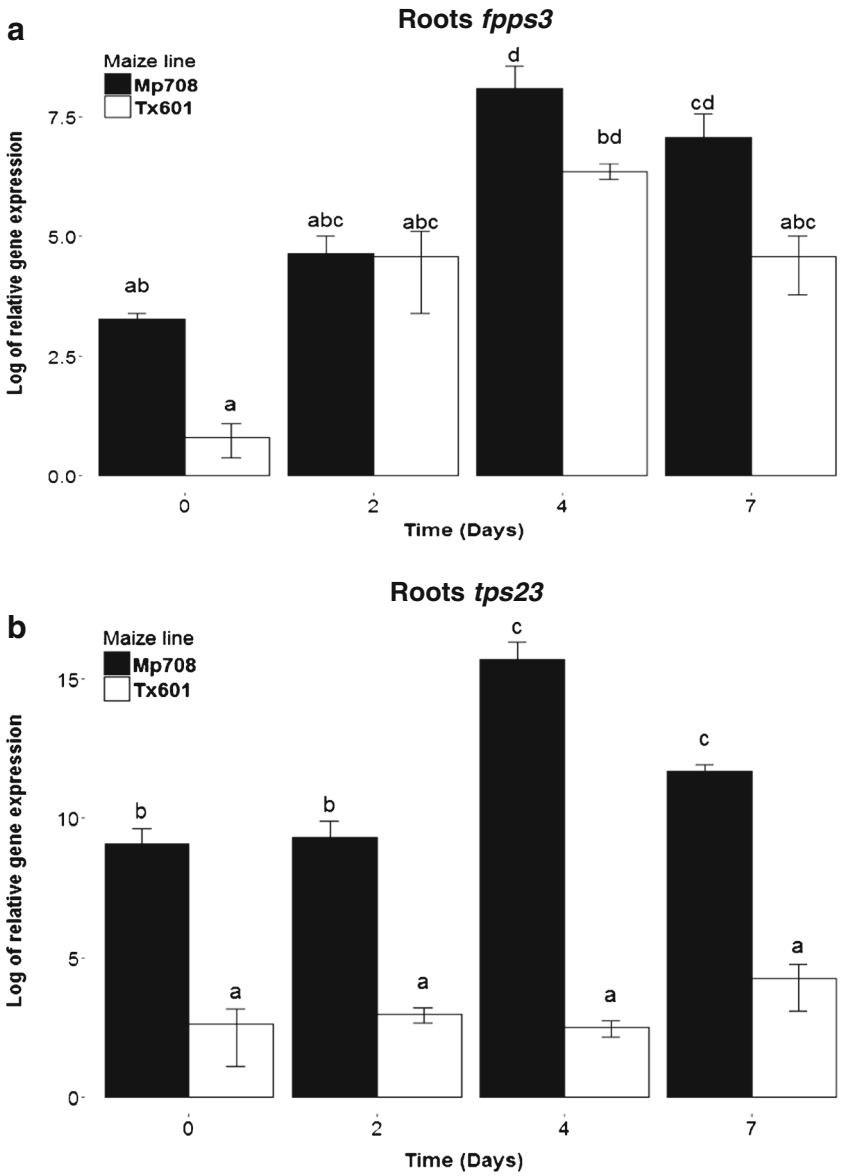

Fig. 7 Time course of maize genes involved in volatile production. **a** *fpps3* and **b** *tps23* transcript accumulation in roots of Mp708 and Tx601 in response to continuous WCR infestation. Gene expression levels were determined in V3 stage plants 0, 2, 4 and 7 d after belowground infestation with WCR. Relative expression (RQ) of *fpps3* and *tps23* were measured by qRT-PCR. Gene expression levels were normalized to *actin*. RQ data were normalized and analyzed using multiple-factor ANOVA followed by honest significant difference (HSD) Tukey pairwise comparison test. Letters represent results of the HSD ($P < 0.05$) and error bars show the standard error. n = 3 per time and per genotype

and RIP2 suggest that Mp708 has a suite of robust defense traits in Its roots. As a result of these root traits, WCR-resistant maize could harbor fewer WCR adults in the field compared to maize genotypes with smaller roots and compromised root growth during belowground infestation (Branson et al. 1982).

Many studies have shown that feeding by chewing insects increases the expression of genes involved in JA biosynthesis and accumulation (Koo and Howe 2009), but the expression of these genes and accumulation of JA in roots during root herbivore attack has not been studied extensively in maize (Erb et al. 2009, 2012). We showed that *aos* transcript levels in Mp708 roots increased up to day 4 of WCR infestation while in Tx601 they remained lower and relatively constant (Fig. 4a). Transcript levels for *opr7* were higher in Mp708 and remained high throughout the infestation whereas those in Tx601 were initially low and only increased slightly during the infestation (Fig. 4b). Notably, the constitutive expression of these two genes was significantly higher in Mp708 than Tx601 roots, similar to higher constitutive expression of these two genes in leaves of older Mp708 plants (Shivaji et al. 2010). The constitutive and induced expression of *aos* and *opr7* may contribute to the higher constitutive and inducible levels of JA in Mp708 than in Tx601. These results support the finding that Mp708 plants Are constitutively defended against herbivory (Shivaji et al. 2010). Our results appear to be consistent with studies that linked JA accumulation with high constitutive and inducible gene expression and accumulation of insecticidal proteins (Ankala et al. 2013; Zhu 2010).

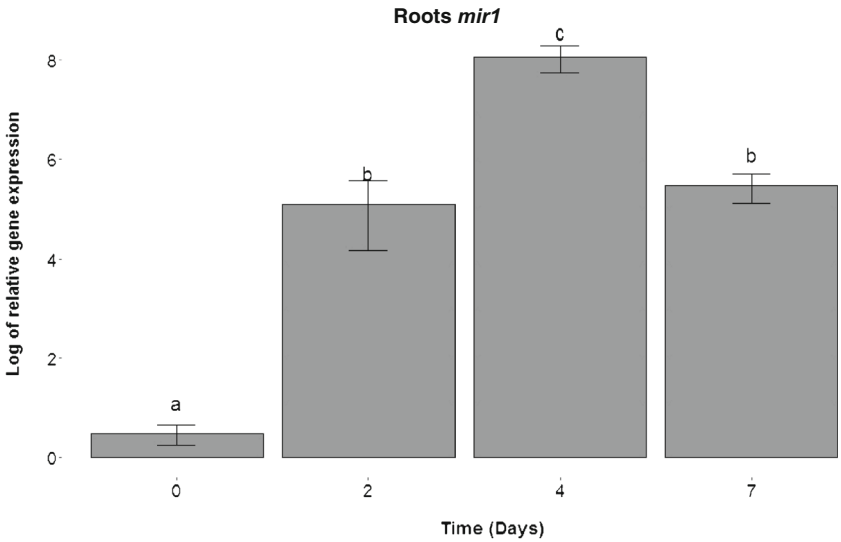

Fig. 8 Time course of *mir1* transcript in roots of Mp708 in response to WCR infestation. Gene expression levels were determined in V3 stage plants 0, 2, 4 and 7 d after belowground infestation with WCR. Relative expression (RQ) of *mir1* was measured by qRT-PCR. Gene expression levels were normalized to *actin*. RQ data were normalized and analyzed using multiple-factor ANOVA followed by honest significant difference (HSD) Tukey pairwise comparison test. Letters represent results of the HSD ($P < 0.05$) and error bars show the standard error; n = 3 per time point

Mp708 roots have high constitutive and inducible levels of JA that suggest it plays a key role in activating downstream defenses against WCR attack.

To understand the downstream molecular changes and production of insecticidal and deterrent molecules involved in Mp708 and Tx601 defense responses to WCR, we examined accumulation of four transcripts, *rip2*, *mpi*, *fpps3*, and *tps23*. Transcripts for *mpi* and *fpps3* significantly increase in both maize lines (Fig. 6b, Fig. 7a) while *rip2* and *tps23* transcripts showed high endogenous and induced levels only in Mp708 (Fig. 6a, Fig. 7b). Because *fpps3* produces FPP (farnesyl pyrophosphate), a precursor of sesquiterpenes, polyphenols, squalene, triterpenes and ubiquinones (Richter et al. 2015; Sallaud et al. 2009), it is possible that both inbreds can increase production of FPP-derived compounds related to plant defenses. Downstream from *fpps3* is *tps23*, a herbivore-induced gene that leads to the production of *(E)*-β-caryophyllene (Kollner et al. 2008), a volatile that attracts entomopathogenic nematodes that Are natural enemies of WCR (Kollner et al. 2008). Mp708, but not Tx601, expressed high constitutive and inducible levels of *tps23* transcripts in the roots (Fig. 7b). This coupled with prior data showing that Mp708 has much higher *(E)*-β-caryophyllene than Tx601 that repelled fall armyworm larvae (Smith et al. 2012) further supports the role of (E)-β-caryophyllene in WCR defense. Thus, the presence of *(E)*-β-caryophyllene could play two important roles in Mp708: deterring fall armyworm feeding and attracting the natural enemies of WCR.

Because diet-based bioassays with WCR Are problematic, we were not able to directly determine the effect of the insecticidal protein MIR1-CP on WCR performance. However, *mir1* transcript levels increased during WCR infestation, implicating MIR1-CP in defense. *Diabrotica* species have a peritrophic matrix (Silva et al. 2004), therefore it is possible that consumption of MIR1-CP by WCR could damage this structure as it does in fall armyworm (Pechan et al. 2000), contributing to plant insect resistance. Our results indicate that both Mp708 and Tx601 use the products of *mpi* and *fpps3* to defend against herbivory, whereas *rip2*, *tps23* and *mir1* Are only inducible in Mp708 and could be key players in Its resistance.

It appears that Mp708 has multiple resistant traits against WCR infestation in addition to other insects with different feeding behaviors like fall armyworm (Williams et al. 1985, 1990) and corn leaf aphid (Louis et al. 2015). Mp708 was developed from landraces of maize that most likely originated in Mesoamerica (Williams et al. 1987), where many phytophagous maize pests including *Diabrotica* sp., have originated (de Lange et al. 2014). One could speculate that multiple generations of selection for adequate yield despite intense insect pressure led to the loss (Moore and Johnson 2017) or incorporation of multiple resistance traits into these landraces, which ultimately were incorporated into Mp708 by selective breeding. Hence, Mp708 displays a suite of resistance traits that encompass both constitutive and inducible defense responses to three types of insect pests: a whorl feeder (fall armyworm), phloem feeder (corn leaf aphid) and root feeder (WCR). Because populations of WCR Are developing resistance to Bt-transgenes (Flagel et al. 2015; Gassmann 2012), soil-applied insecticide and persist despite crop rotation with soybean (Bigger 1932; Gray et al. 2009), the availability of a non-transgenic genotype with this remarkable range of native host plant resistance will be especially useful for discovering new resistance traits that can be implemented in plant breeding and pest management programs against a highly adaptable insect like WCR.

Acknowledgements This material is based upon work supported by the National Science Foundation Graduate Research Fellowship Program under Grant No. DGE1255832. We would like to thank Dr. Wade French and Chad Nielson from USDA-ARS in Brookings, SD for sending us WCR eggs.

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