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Fighting on two fronts: Elevated insect resistance in flooded maize

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Abstract

To grow and thrive plants must be able to adapt to both adverse environmental conditions and attack by a variety of pests. Elucidating the sophisticated mechanisms plants have developed to achieve this has been the focus of many studies. What is less well understood is how plants respond when faced with multiple stressors simultaneously. In this study, we assess the response of Zea mays (maize) to the combinatorial stress of flooding and infestation with the insect pest Spodoptera frugiperda (fall armyworm). This combined stress leads to elevated production of the defence hormone salicylic acid, which does not occur in the individual stresses, and the resultant salicylic acid-dependent increase in S. frugiperda resistance. Remodelling of phenylpropanoid pathways also occurs in response to this combinatorial stress leading to increased production of the anti-insect C-glycosyl flavones (maysins) and the herbivore-induced volatile phenolics, benzyl acetate, and phenethyl acetate. Furthermore, changes in cellular redox status also occur, as indicated by reductions in peroxidase and polyphenol oxidase activity. These data suggest that metabolite changes important for flooding tolerance and anti-insect defence may act both additively and synergistically to provide extra protection to the plant.

KEYWORDS
fall armyworm, maysin, phenylpropanoid, salicylic acid, submergence, volatiles, Zea mays

1 | INTRODUCTION

Maize (Zea mays) is a key global agricultural commodity, accounting for the majority of animal feedstocks, while also being important for biofuel production, human nutrition, and dozens of industrial applications. Although maize breeding and technology have resulted in unprecedented yields, biotic challenge from pathogens and insect pests continues to cause major losses, with an estimated 6–19% of global production lost due to insects and other herbivores (Oerke, 2006) and around 10% to diseases (Mueller et al., 2016). Further exacerbating this problem, abiotic stresses such as temperature extremes and drought also have major effects on yield. For example, in 2012, when the combined effects of extreme heat and drought were coupled with an outbreak of corn rootworm (Diabrotica virgifera virgifera) and increased fungal infection, it led to the lowest harvest per unit area planted in over 20 years in the United States (Boyer et al., 2013).

The problem of combined abiotic and biotic stresses is complicated by the fact that the response of maize to an abiotic stress can alter its ability to respond to biotic attack. For example, elevated atmospheric carbon dioxide levels increases the susceptibility of maize to the fungal pathogen Fusarium verticillioides (Vaughan et al., 2014). Furthermore, this susceptibility is amplified if the plants are exposed to both elevated carbon dioxide and drought (Vaughan et al., 2016). Abiotic stresses such as drought have also been shown to impact the production of compounds in maize involved in disease resistance (Vaughan et al., 2015), whereas elevated carbon dioxide impedes the ability of maize to produce defences against herbivory (Block, Vaughan, Christensen, Alborn, & Tumlinson, 2017). Furthermore, studies in other plant species have shown that different abiotic stresses can have positive or negative effects on insect resistance (Suzuki, Rivero, Shulaev, Blumwald, & Mittler, 2014). Oftentimes, these antagonistic or synergistic effects can be traced back to function of hormones in regulating the specific stress responses (Nguyen, Rieu, Mariani, & van Dam, 2016).

An abiotic stress of maize that has the potential to impact pest resistance is flooding (submergence and/or waterlogging of soils). Maize is sensitive to flooding stress, particularly at the seedling stage, with some susceptible cultivars dying after just 24 hours of waterlogging, whereas more resistant cultivars can tolerate submergence for 2 weeks or more. All cultivars tested show yield losses after extended periods of flooding (Tang, Xu, Zou, Zheng, & Qiu, 2010). The anoxia in the roots caused by flooding leads to an increased accumulation of reactive oxygen species and changes in the production of cell wall-modifying enzymes that likely play a role in the formation of aerenchyma (specialized structures to facilitate gas exchange in flooded roots) and adventitious roots (Rajhi et al., 2011; Thirunavukkarasu et al., 2013; Yu et al., 2015). The major metabolic and physiological changes that occur in maize in response to flooding may affect the ability of the plant to defend itself against biotic attack. We therefore examined the effect of flooding on the response of maize seedlings to attack by the folivorous insect pest Spodoptera frugiperda (fall armyworm). We observed that flooded plants were more resistant to S. frugiperda and that this resistance was associated with an unexpected increase in the plant hormone salicylic acid that is not known to be a major regulator of biotic defences in maize.

2 | MATERIAL AND METHODS

2.1 | Larval growth assays

For the S. frugiperda assays, 2-week-old B73 maize plants were placed in a flooding pool with the waterline 1 cm over soil level or as nonflooded controls, on a bench in the same greenhouse (20 plants for each treatment). Three days later, five S. frugiperda neonates were placed into the whorl of each plant. Larval weights were determined daily on Days 3–7 post infestation. Due to some larval loss due to death of neonates or cannibalism/injury of larvae, a total of 60–100 larvae were weighed for each treatment at each time point. Larvae from each treatment were retained on the treated plants by placing the plants in insect cages. Plants were removed from the flooding pool and relocated into insect cages on the bench on the fourth day after infestation and remained unsubmerged for the rest of the experiment. Larval growth assays were repeated at least three times.

2.2 | Metabolite analysis

Maize tissue was collected at the specified times post infestation and rapidly frozen in liquid nitrogen. Tissue was sampled from a 12-cm^2 area of an inner whorl leaf from each plant, and leaves/sections were chosen that displayed both evidence of multiple feeding sites and active larval feeding. Noninfested samples were taken from the corresponding leaf sections on uninfested plants, each plant was used for a single sample, and four samples were taken for each treatment at each time point. For analysis of the phytohormones jasmonic acid and salicylic acid and the metabolites benzoic acid and cinnamic acid, metabolites were extracted, derivatized, and quantified by gas chromatography–mass spectrometry (GC-MS) using the vapour phase extraction method as described in Schmelz, Engelberth, Tumlinson, Block, and Alborn (2004). Analysis of benzoxazinoids was performed using liquid chromatography–mass spectrometry as described in Block, Hunter, Rering, Christensen, and Meagher (2018).

Volatile emission was determined by removing plants from the flooding conditions at 4 p.m. 3 days post infestation and placing them in volatile collection chambers (Volatile collection system model TVCSx4V, Analytical Research Systems, Gainesville, FL, USA). Volatiles were collected on SuperQ columns for 20 hr with an incoming air flow of 1.7 L min^{-1} and a vacuum of 0.7 L min^{-1}. At the end of the collection, an internal standard of 2 μg of nonyl acetate was added to the SuperQ columns, and volatiles were eluted from the columns with 150 μl of methylene chloride and analysed by GC-MS, as described in Block et al. (2017).

Maysins were extracted from 100 mg of tissue by homogenization in 0.5 ml of extraction buffer (methanol:glacial acetic acid [98:2]) containing 50 μg of the maysin surrogate crysin. Homogenized tissue was sonicated for 20 min in extraction buffer and centrifuged at 16,000g for 10 min. The resulting supernatant was filtered through a 0.22-μm PVDF membrane and 5 μl of each sample analysed by high performance liquid chromatography–diode array spectrophotometry (HPLC-DAD). Maysins were separated on a Waters Symmetry®C18 35 μm × 4.6 mm × 100 mm column with a Vanguard Symmetry C18 pre-column using the following solvents: Solvent A (water with 0.1% formic acid) and Solvent B (acetonitrile with 0.1% formic acid), run at 1 min with Solvent A:Solvent B (95:5) and then switching to 100% Solvent B over a 20-min time frame. Maysins were monitored at 350 nm using a 550-nm reference wavelength. Crysin was eluted at 19.06 min and various maysins between 10.2 and 14.5 min. As maysins are not commercially available, validation of peaks was achieved by comparison of extracts from maysin-rich sweetcorn variety Zalpalote Chico (Snook, Widstrom, & Gueldner, 1989) and maysin-deficient "salmon silks" maize mutants (Casas et al., 2016).

2.3 | Phenolic analysis

Soluble phenolic components were extracted from 100 mg of B73 maize seedlings as described in Sarath, Baird, Vogel, and Mitchell (2007). Briefly, soluble phenolic components were extracted using 1.5% acetic acid in 50% methanol (v/v). Wall-bound phenolics were extracted using residual plant material suspended in 4.0M NaOH incubated at 90°C for 2 hr, and released phenolics were extracted into ethyl acetate after acidification with 6.0M HCl. Ethyl acetate extracts were vacuum-dried, each extract (soluble and wall-bound) was derivatized with trimethylsilyl (TMS) and trifluoroacetamide (MSTFA; Thermo Fisher), and toluic acid was included in this reaction as an internal standard for quantification. The products were analysed using GC-MS. Relative abundances of soluble and wall-bound phenolic compounds were determined by the peak areas of major ions. Between-sample normalization was performed using the peak area for the internal standard, toluic acid. Analysis was performed in duplicates on five biological replicates per treatment.

2.4 | NPR1 phylogenetic analysis and mutant identification

Predicted protein sequences for NPR1-related genes were identified from BLASTP queries of gene databases for maize (Ensembl-18), rice (v7_JGI), and Arabidopsis (TAIR10) conducted on phytozome (JGI). Cut-off for inclusion in phylogenetic analysis was e-value greater than 1e−6. A MUSCLE alignment (Edgar, 2004) of predicted protein sequences from the primary transcript of each gene was used to construct a phylogeny using the neighbour-joining method based on the Tamura-Nei model (Tamura & Nei, 1993) with no outgroup and 1,000 random-seeded bootstrap repetitions. Accession numbers for the genes analysed are ZmNPR1 (GRMZM2G077197), ZmNPR2 (GRMZM2G076450), ZmNPR3 (GRMZM2G115162), ZmNPR-like1 (GRMZM2G026556), ZmNPR-like2 (GRMZM2G022606), ZmBOP1-like1 (GRMZM2G060723), ZmBOP1-like2 (GRMZM2G039867), AtNPR1 (AT1G64280), AtNPR2 (AT4G26120), AtNPR3 (AT5G45110), AtNPR4 (AT4G19660), AtBOP1 (AT3G57130), AtBOP2 (AT2G41370), OsBTBA1/NPR1 (LOC_Os01g09800), OsBTBA2 (LOC_Os01g56200), OSBTBA3 (LOC_Os01g72020), OsBTBA4 (LOC_Os03g46440), OsBTBA5 (LOC_Os11g04600), and OsBTBA6 (LOC_Os12g04410; Figure S2a).

The npr1 mutant was identified in the UniformMu line UFMu-08697 (McCarty et al., 2013) obtained from the Maize Genetics Cooperation Stock Center (maizecoop.cropsci.uiuc.edu). The line contained a Mutator transposable element (mu1067056) in the predicted NPR1-coding maize ortholog ZmNPR1 (GRMZM2G077197). The insertion was confirmed to be present in the predicted coding sequence of the first exon of ZmNPR1 (Figure S2b) using the transposon-specific PCR primer TIR6 in combination with gene-specific primers for ZmNPR1 (Table S1). A homozygous mutant line was established by self-pollinating a genotyped, homozygous mutant. All assays with the npr1 mutant line were performed using the parental bronze-1 W22 inbred (bronze-1 mu-mutatble-9) fixed as homozygous after nine generations of introgression as the wild-type control. All other assays were conducted in the B73 inbred background.

2.5 | Gene expression analysis

Gene expression was induced in 2-week-old B73 plants treated as in the larval growth assays, with tissue collected at 2 days post infestation of both infested and control plants. RNA extraction and qRT-PCR were performed as in Block et al. (2018) using the geometric mean of the reference genes folylpolyglutamate synthase (FPGS, GRMZM2G393334) and ubiquitin carrier protein (UBCP, GRMZM2G102471; Manoli, Sturaro, Trevisan, Quaggiotti, & Nonis, 2012) and normalizing to the noninfested, nonflooded control. The gene-specific primers used are listed in Table S1.

2.6 | Protein quantification and enzyme assays

For the quantification of total soluble proteins, leaves were ground with pestle and mortar in liquid nitrogen. Aliquots of the resulting powder (35–75 mg) were thawed and homogenized in 50-mM KH_2PO_4/KOH pH 7.0, 150-mM KCl, and 3% (w/v) crossed-linked polyvinylpryrrolidone using a glass-rod potter. Extracts were then clarified by centrifugation (18,000g for 5 min at 4°C), and proteins were quantified in the supernatants by the method of Bradford (1976) using BSA as a standard. Peroxidase activity was determined on total protein extract from B73 leaf tissue, as described in Baaziz and Saaldl, by providing the substrates guaiacol and hydrogen peroxide and monitoring the change in absorbance at 470 nm due to the formation of tetraguaiacol (Baaziz & Saaidi, 1988). Polyphenol oxidase activity was determined on total protein extract, as described in Siddiq et al., by monitoring the oxidation of catechol at 420 nm (Siddiq, Sinha, & Cash, 1992).

2.7 | Statistical analysis

All statistical analyses were performed using Microsoft Excel. Pairwise comparisons were determined using t tests assuming equal variances, and multifactorial comparisons were assessed using ANOVA coupled with multiple pairwise comparisons. Post hoc testing of normality and equal variance were done by assessing skewness and running F tests, respectively. All measurements that required different sample processing and analysis were taken from different sets of plants/treatments. For gene expression analysis, the relative quantities were calculated using the $2^{-\Delta\Delta Ct}$ method, and statistical tests were run on the transformed data. Samples were statistically significant with a $P \leq .05$.

3 | RESULTS

3.1 | Flooding of maize causes a salicylic acid-mediated increase in insect resistance

To examine the impact of flooding on the resistance of maize seedlings to the leaf-chewing caterpillar S. frugiperda, maize plants under flooded or nonflooded conditions were infested with S. frugiperda neonates. Measurement of larval growth on flooded versus control plants revealed that S. frugiperda had a significantly slower growth rate on flooded maize than on nonflooded controls (Figure 1a). One possible reason for the reduced growth of S. frugiperda larvae on flooded maize is an altered nutritional content of the maize leaves, as flooding

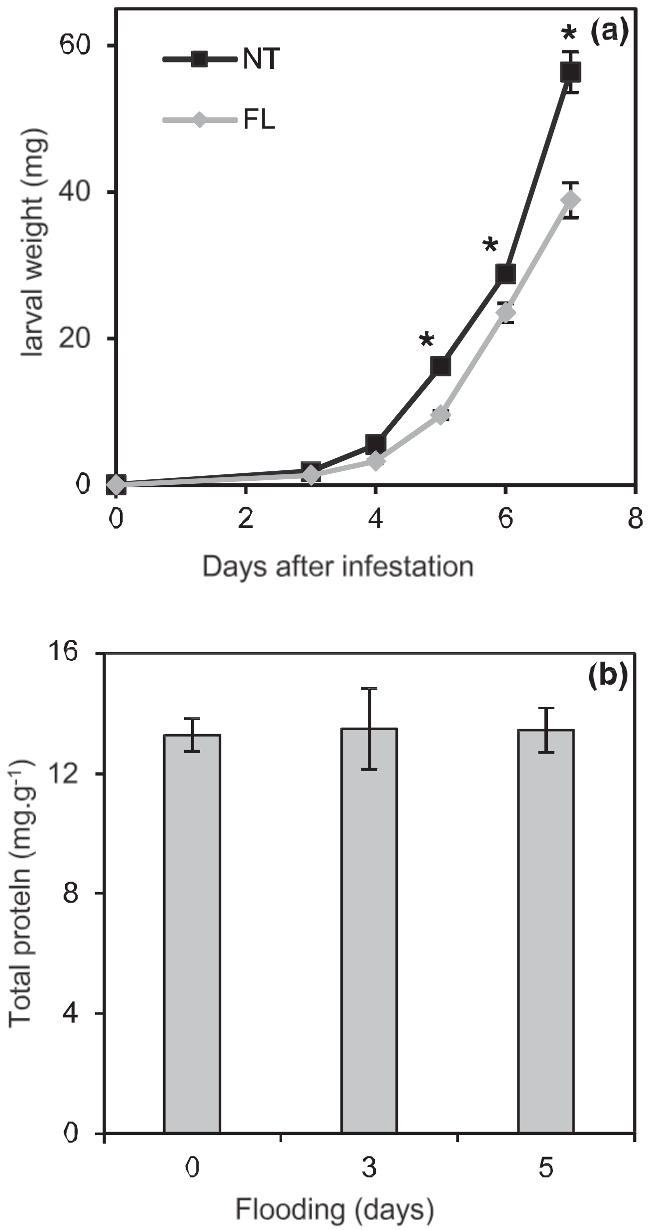

FIGURE 1 Flood-induced Spodoptera frugiperda resistance in maize not due to altered protein levels. (a) Growth of S. frugiperda on nontreated (NT) and flooded (FL) maize plants (n = 60, bars = mean ± SEM). (b) Total protein in maize leaf tissue from flooded plants at 0, 3, and 5 days after flooding (n = 6, bars = mean ± SEM). Treatments significantly different from NT, $P \leq .05$ by t test, indicated by *

impacts maize development. To investigate possible changes to the nutritional content of maize during flooding, the total protein content was measured in maize leaves after 0, 3, and 5 days of flooding (Figure 1b). No significant differences in total protein content were observed in maize leaves over the period examined, suggesting that the observed negative effect on larval growth in flooded plants may result more from altered maize defences than from nutritional changes.

A variety of anti-herbivore defences in maize are induced by the phytohormone jasmonic acid, which is upregulated in response to herbivory. To examine if flooding impacted S. frugiperda induced jasmonic acid production, levels of this phytohormone were measured in infested plants under flooded and nonflooded conditions for 4 days (Figure 2a). No significant differences in jasmonic acid production were observed between infested plants with or without flooding at any of the time points examined. These data demonstrate that the enhanced resistance seen in flooded plants is not due to increased jasmonic acid production.

Another strong regulator of plant defences in many species is salicylic acid. Interestingly, flooded plants accumulated salicylic acid to levels above 1 μg g^{-1} fresh weight at 4 days post S. frugiperda infestation (Figure 2b). Comparatively, nonflooded plants did not accumulate heightened levels of salicylic acid in response to S. frugiperda

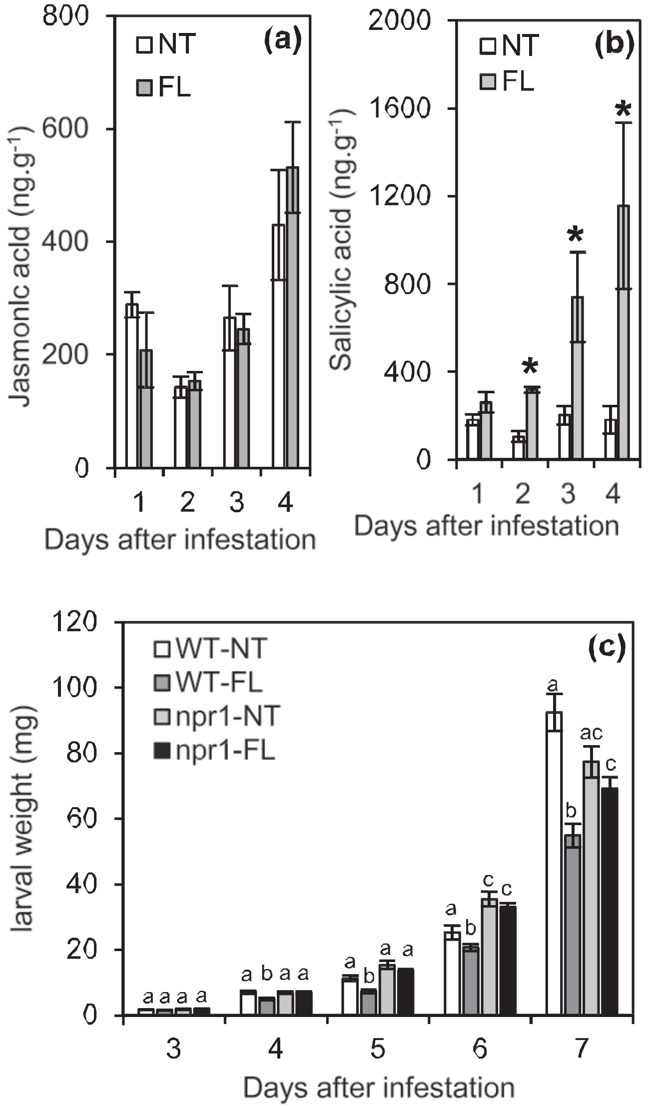

FIGURE 2 Elevated salicylic acid important for flood-induced resistance to Spodoptera frugiperda in maize. Nontreated (NT) and flooded (FL) maize plants were infested with S. frugiperda, and (a) jasmonic acid and (b) salicylic acid were measured at 1–4 days after infestation, n = 4, bars = mean ± SEM. Treatments significantly different from NT, $P \leq .05$ by t test, indicated by *. (c) Growth of S. frugiperda on NT and FL wild-type (WT) and salicylic acid receptor mutant (npr1) maize plants (n = 60, bars = mean ± SEM). Letters indicate significantly different treatments $P \leq .05$ by ANOVA

infestation; therefore, salicylic acid levels were significantly higher in flooded plants compared with nonflooded plants from 2 days post infestation onwards. To examine whether flooding alone leads to accumulation of salicylic acid, levels were quantified in flooded and nonflooded plants that had not been infested by S. frugiperda (Figure S1a). No significant accumulation of salicylic acid was observed due to flooding alone. These data show that the combined stresses of flooding and S. frugiperda infestation led to accumulation of salicylic acid in maize.

To determine if this increased production of salicylic acid was important for flood-induced S. frugiperda resistance, a loss-of-function mutant was isolated in the maize homolog of the salicylic acid receptor NPR1 (Figure S2), and S. frugiperda growth was compared on flooded and nonflooded wild-type and npr1 mutant plants (Figure 2c). The wild-type control plants displayed a significant reduction in S. frugiperda growth in flooded plants compared with the nonflooded controls, whereas flooding had no significant impact on the growth of S. frugiperda larvae on npr1 mutant plants. These data support the hypothesis that the accumulation of salicylic acid plays a role in flood-induced resistance.

3.2 | The combination of flooding and S. frugiperda infestation alters the phenylpropanoid profile of maize

Structural changes in maize that occur during flooding involve modification of phenylpropanoid-derived cell wall components. This coupled with the role of salicylic acid in flood-induced insect resistance, and a potential biosynthesis pathway of salicylic acid from phenylpropanoids, led us to examine possible changes in phenylpropanoid metabolism caused by this combinatorial stress. To examine this, the levels of benzoic acid and cinnamic acid were measured in flooded or nonflooded plants infested with S. frugiperda (Figure 3a,b). Similarly to salicylic acid, both benzoic acid and cinnamic acid were induced in response to the combined stresses of flooding and S. frugiperda infestation, reaching levels in flooded plants significantly higher than in nonflooded plants from 2 days post infestation onwards. Like salicylic acid, benzoic acid was not induced in response to the individual stresses (Figure S1b), whereas cinnamic acid was induced in response to both flooding and herbivory in isolation and the combined stress had an additive effect on its production (Figure S1c).

To further examine the effects of flooding on phenylpropanoids in infested plants, levels of soluble and wall-bound phenolics were determined in flooded and nonflooded plants that were either noninfested or infested for 2 days with S. frugiperda (Figure 3c–f). Consistent to what was seen in Figure S1b, levels of soluble benzoic acid marginally increased in response to the combined stress of flooding and S. frugiperda infestation but not to the individual stresses (Figure 3d); however, levels of wall-bound benzoic acid were unchanged (Figure 3f). In contrast, the quantity of soluble 4-coumaric acid increased in response to S. frugiperda infestation irrespective of flooding (Figure 3c), whereas wall-bound 4-coumaric acid increased only in response to combined flooding and infestation (Figure 3e).

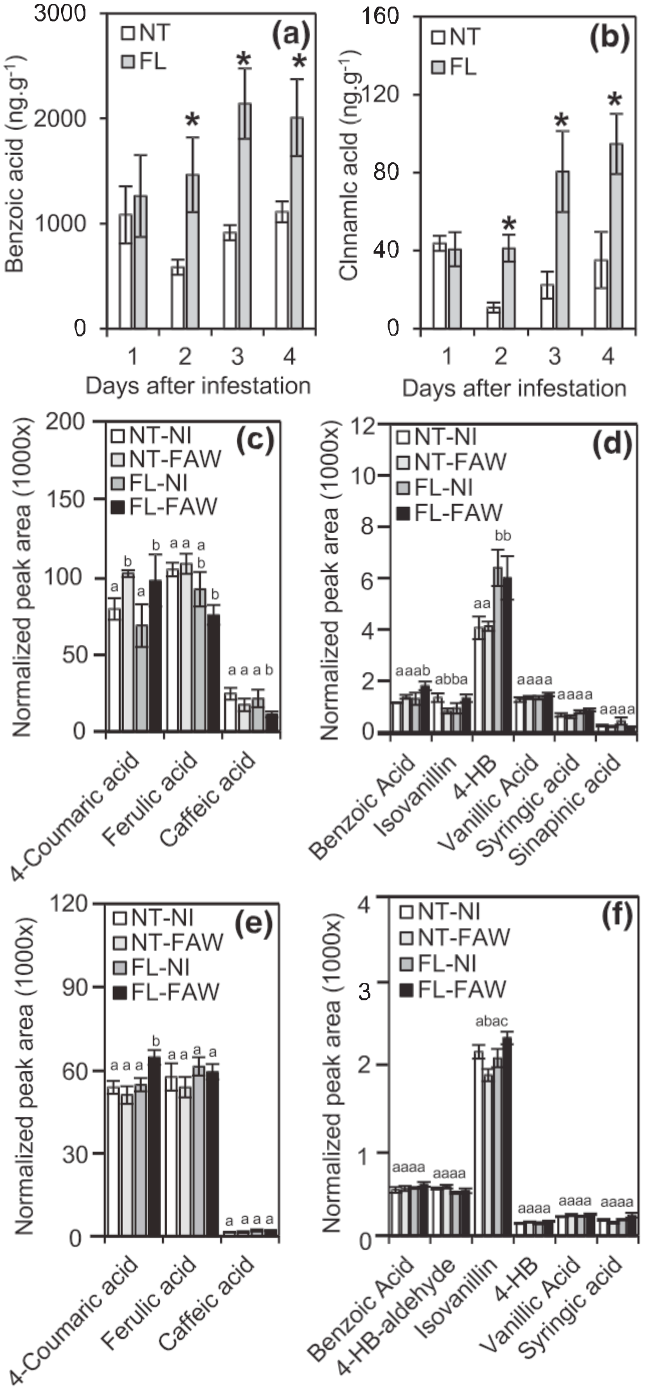

FIGURE 3 Phenylpropanoid remodelling during Spodoptera frugiperda infestation of flooded maize. Nontreated (NT) and flooded (FL) maize plants were infested with S. frugiperda, and (a) benzoic acid and (b) cinnamic acid measured at 1–4 days after infestation, n = 4, bars = mean ± SEM. Treatments significantly different from NT, $P \leq .05$ by t test, indicated by *. NT and FL maize plants were noninfested (NI) or infested with S. frugiperda (FAW), and (c, d) soluble and (e, f) wall-bound phenolics measured at 2 days after infestation, 4-hydroxybenzaldehyde (4-HB-aldehyde), 4-hydroxybenzoic acid (4-HB), n = 5, bars = mean ± SEM. Letters indicate significantly different treatments, $P \leq .05$, by ANOVA

Soluble levels of both ferulic and caffeic acid decreased in response to combined flooding and infestation (Figure 3c), whereas wall-bound levels were unaltered (Figure 3e). Furthermore, soluble but not wall-bound levels of 4-hydroxybenzoic acid increased in response to flooding (Figure 3d,f), whereas soluble and wall-bound isovanillin levels decreased in nonflooded infested plants (Figure 3d,f). Soluble levels of vanillic acid, syringic acid, and sinapinic acid were unchanged (Figure 3d) as were wall-bound levels of 4-hydroxybenzaldehyde, vanillic acid, and syringic acid (Figure 3f). These changes are marginal when compared with the large increases in salicylic acid, yet they suggest that highly specific remodelling of phenylpropanoid metabolism occurs in response to flooding and infestation.

To assess if salicylic acid was involved in this phenylpropanoid remodelling, the induction of benzoic acid and cinnamic acid by flooding was assessed in S. frugiperda-infested npr1 mutant plants (Figure 4). There was no significant difference in the production of either benzoic acid or cinnamic acid in infested npr1 plants due to flooding. This is in sharp contrast to the wild-type plants that show a significant increase in both compounds due to flooding when infested. These data suggest that the flooding-induced production of cinnamic acid and benzoic acid is likely dependent upon salicylic acid.

3.3 | Flooding enhances S. frugiperda-induced emissions of specific volatiles

In addition to salicylic acid and soluble and wall-bound phenolics, maize also produces volatile phenolics including benzyl acetate and phenethyl acetate, which are likely derived from benzoic acid and cinnamic acid, respectively. These compounds are induced in response to herbivory. To determine if flooding impacts the production of these and other S. frugiperda-induced volatiles, emission of volatiles was measured in flooded and nonflooded plants with and without S. frugiperda infestation (Figure 5). Emission of the monoterpene volatiles (beta-myrcene and beta-ocimene) as well as the sesquiterpene volatiles (beta-caryophyllene, alpha-bergamotene, beta-farnesene, and germacrene D) was strongly induced by S. frugiperda infestation to the same extent in both flooded and nonflooded plants (Figure 5a). The same was true for indole and the homoterpene volatile (3E)-4,8-dimethyl-l,3,7-nonatriene (DMNT). Therefore, these herbivore-induced volatiles were not impacted by flooding.

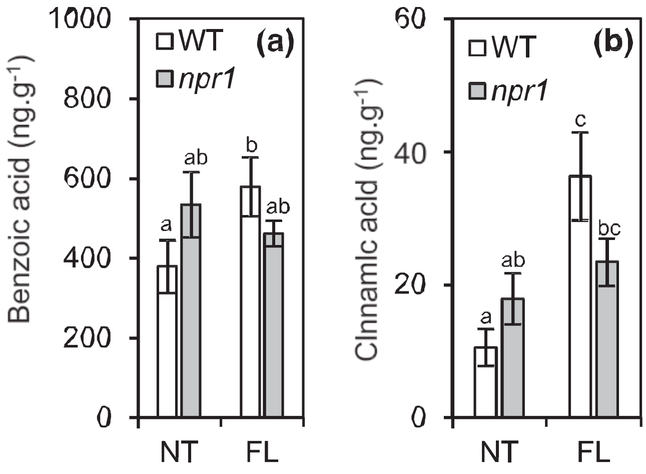

FIGURE 4 Flooding of infested plants enhances the production of benzoic acid and cinnamic acid in a salicylic acid-dependent manner. (a) Benzoic acid and (b) cinnamic acid were measured in nontreated (NT) and flooded (FL) wild-type (WT) or salicylic acid receptor mutant (npr1) plants infested with S. frugiperda at 2 days after infestation, n = 6, bars = mean ± SEM. Letters indicate significantly different treatments, P ≤ .05, by ANOVA

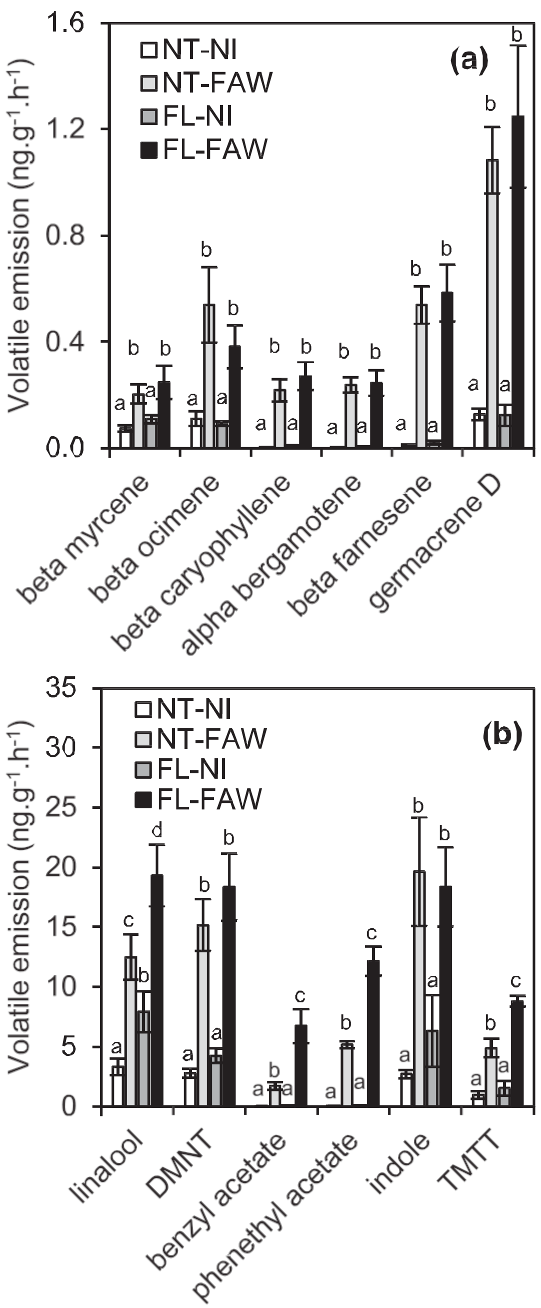

FIGURE 5 Flooding alters production of specific herbivore-induced volatiles. Nontreated (NT) and flooded (FL) maize plants were noninfested (NI) or infested with Spodoptera frugiperda (FAW) and volatile emission measured for 20 hr 3 days post infestation, (a) low abundance volatiles, (b) high abundance volatiles, (3E)-4,8-dimethyl-l,3,7-nonatriene (DMNT), (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), n = 4, bars = mean ± SEM. Letters indicate significantly different treatments, P ≤ .05, by ANOVA

In contrast, the homoterpene (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) and the monoterpene alcohol linalool were significantly higher in S. frugiperda-infested plants that were flooded compared with nonflooded plants (Figure 5b). Linalool production was also significantly higher in flooded than nonflooded plants without S. frugiperda infestation indicating that its increased production may be solely due to flood-related stresses. The volatile phenolics, benzyl acetate, and phenethyl acetate were induced in response to S. frugiperda infestation to significantly higher levels in flooded than in nonflooded plants but showed no difference due to flooding in noninfested plants (Figure 5b). These data show that the production herbivore-induced volatiles TMTT, linalool, and volatile phenolics are specifically enhanced by the combination of flooding and S. frugiperda infestation.

3.4 | The combination of flooding and S. frugiperda infestation increases the production of anti-herbivore maysins in a salicylic acid independent manner

The observed remodelling of phenylpropanoid metabolism by the combined stress of flooding and S. frugiperda infestation suggests the potential for enhanced insect resistance due to the production of a family of anti-insect C-glycosyl flavones in maize, collectively termed maysins. Indeed, two of the phenylpropanoids, cinnamic acid, and 4-coumaric acid, which are induced by this combinatorial stress, are precursors of maysins. Total production of maysins was therefore examined in maize leaf tissues 4 days after S. frugiperda infestation (Figure 6a). The combination of flooding and S. frugiperda infestation led to a significantly approximately twofold increase in the accumulation of maysins compared with the nontreated controls. Interestingly, S. frugiperda infestation in nonflooded plants led to a significant reduction in maysins compared with the noninfested controls, whereas flooding treatment alone had no significant impact on the production of maysins. These data suggest that the combinatorial stress of flooding and S. frugiperda infestation leads to increased production of the anti-insect maysins, which could contribute to the increased resistance to S. frugiperda.

To determine if salicylic acid was involved in regulating the observed increase in maysins, the levels of maysins in flooded and nonflooded npr1 mutant plants and their corresponding wild-type plants were assessed 3 days post infestation with S. frugiperda (Figure 6b). Both wild-type and npr1 mutant plants accumulated elevated levels of maysins in response to the combined stress of flooding and S. frugiperda infestation compared with nonflooded controls, with no significant difference in maysin levels between the two lines in either treatment tested. These data indicate that the increased maysin levels in flooded infested plants are not dependent on salicylic acid-based signalling mediated by NPR1.

To investigate whether other metabolites known to be important in insect resistance were induced during combined flooding and S. frugiperda infestation, the production of indole-derived benzoxazinoids was examined (Figure 6c). Levels of 2,4,7-trihydroxy-2H-1,4-benzoxazin-3-(4H)-one-glc (TRIBOA-Glc), 2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one (HBOA-Glc), 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-Glc), 2-β-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA-Glc), and 2-β-D-glucopyranosyloxy-4-hydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one (DIM2BOA-Glc) were unaffected by either flooding or herbivory. 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-Glc)

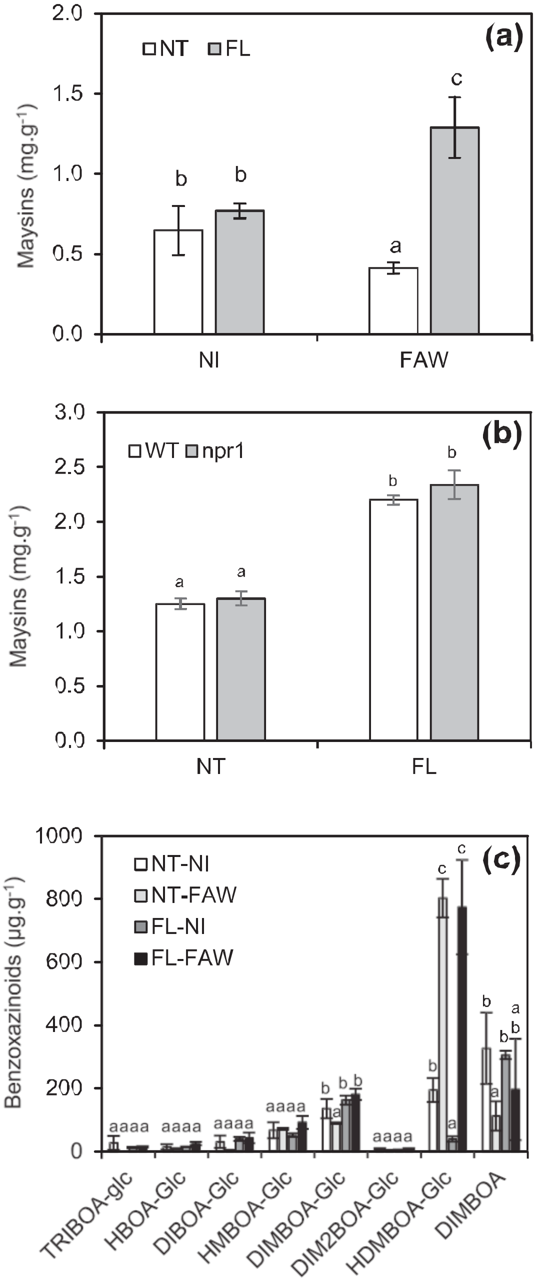

FIGURE 6 Flooding of infested plants enhances the production of maysins but not benzoxazinoids in a salicylic acid-independent manner. (a) Maysins were measured in nontreated (NT) and flooded (FL) wild-type maize plants that were noninfested (NI) or infested with Spodoptera frugiperda (FAW) at 2 days after infestation, (b) maysin was measured in NT or FL wild-type (WT) or salicylic acid receptor mutant (npr1) plants infested with FAW, and (c) benzoxazinoids were measured in NT or FL wild-type plants NI or infested with FAW, n = 4, bars = mean ± SEM. Letters indicate significantly different treatments, $P \leq .05$, by ANOVA

was slightly but significantly reduced in S. frugiperda-infested nonflooded plants compared with flooded and noninfested plants. 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) was reduced in nonflooded S. frugiperda-infested plants compared with nonflooded or flooded noninfested plants. However, it was not significantly lower than flooded infested plants suggesting that flooding does not significantly impact this metabolite. 2-Hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc), the most abundant of the benzoxazinoids examined in infested plants, was significantly induced by infestation. There was no difference in HDMBOA-Glc levels between infested flooded and infested nonflooded plants though it was significantly lower in flooded noninfested plants than in nonflooded noninfested plants. These data show that flooding does not significantly impact benzoxazinoid accumulation in infested plants but does change the benzoxazinoid profile in noninfested plants. Overall, these data show that the effect of the combined stress of flooding and S. frugiperda infestation specifically increases the production of maysins rather than nonspecifically upregulating anti-insect defences.

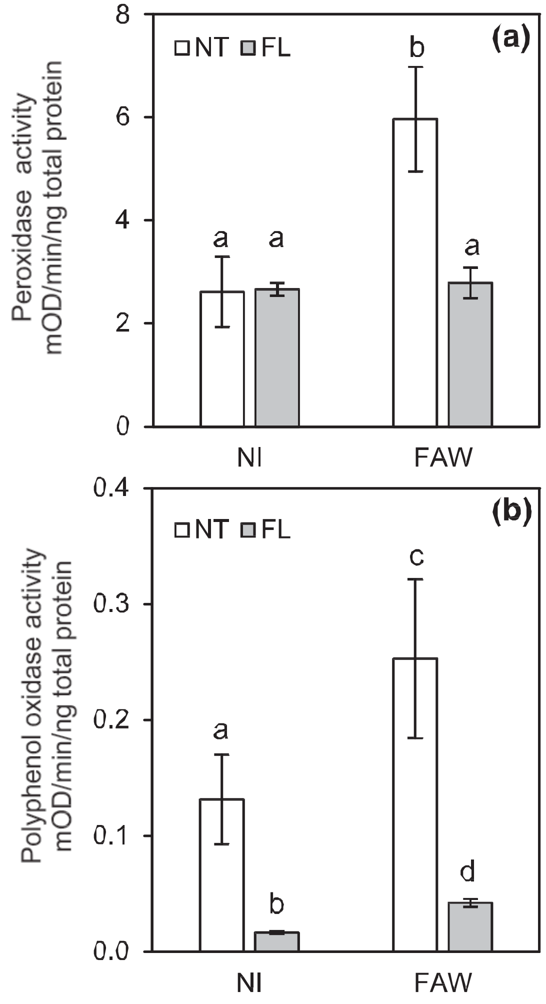

FIGURE 7 Impact of Spodoptera frugiperda infestation of flooded maize on peroxidase and polyphenol oxidase activity. Nontreated (NT) and flooded (FL) maize plants were infested with S. frugiperda (FAW) or not infested (NI) and enzyme activity measured at 2 days after infestation, (a) peroxidase activity, (b) polyphenyl oxidase activity, n = 4, bars = mean ± SEM. Letters indicate significantly different treatments, P ≤ .05, by ANOVA

3.5 | Flooding suppresses S. frugiperda-induced peroxidase and polyphenol oxidase activities

Infestation of maize with insects causes increased activity of redox related enzymes such as peroxidases and polyphenol oxidases that impact cell wall cross-linking. Treatment of maize with exogenous salicylic acid causes a weak but significant reduction in the activity of soluble guaiacol peroxidase activity (Mika, Boenisch, Hopff, & Luthje, 2010). The effect of flooding on the induction of peroxidase and polyphenol oxidase activity in maize infested with S. frugiperda was therefore assessed. Total soluble peroxidase activity in maize was increased by more than twofold in S. frugiperda-infested plants under nonflooded conditions compared with noninfested controls (Figure 7a). When plants were flooded, the S. frugiperda-associated

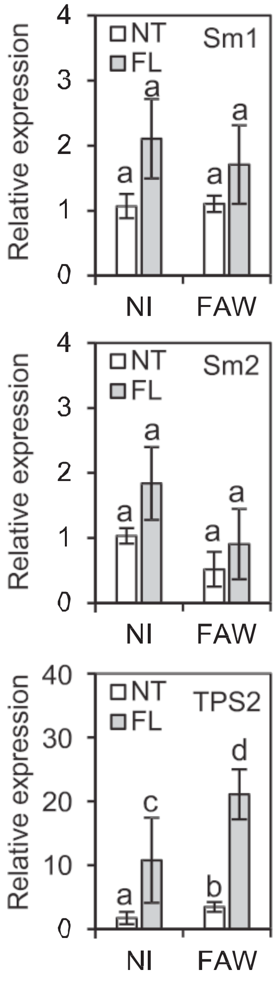

FIGURE 8 Impact of flooding on defence gene expression. Nontreated (NT) and flooded (FL) maize plants were infested with Spodoptera frugiperda (FAW) or not infested (NI) and relative gene expression measured at 4 days after infestation, n = 6, bars = mean ± SEM. Letters indicate significantly different treatments, P ≤ .05, by ANOVA

increase in total peroxidase activity was suppressed, with no significant difference between infested and noninfested plants. Furthermore, no significant difference in peroxidase activity was observed between the flooded and nonflooded noninfested controls, indicating that flooding specifically suppresses the S. frugiperda-induced peroxidase activity.

Polyphenol oxidase activity in maize was also increased around twofold in S. frugiperda-infested plants under nonflooded conditions compared with noninfested controls (Figure 7b). In contrast to peroxidase activity, polyphenol oxidase activity was strongly suppressed by flooding in both infested and noninfested plants to levels significantly below those of noninfested nonflooded controls. Despite this result, flooded plants had higher levels of polyphenol oxidase activity after S. frugiperda infestation. These data indicate that flooding suppresses polyphenol oxidase activity irrespective of S. frugiperda infestation.

3.6 | Changes in gene expression in response to flooding and S. frugiperda herbivory

To investigate whether the metabolic changes observed during the combination of flooding and herbivory were due to changes in specific dedicated metabolic pathways, the expression of representative genes for enzymes involved in the biosynthesis of select defence-related compounds was examined. To determine the impact on genes involved in the production of maysin, the relative expression of genes encoding the last two reactions of maysin biosynthesis, Sm1 (GRMZM2G031311) and Sm2 (GRMZM2G180283; Casas et al., 2016), was analysed (Figure 8). Neither Sm1 nor Sm2 were induced in response to herbivory by S. frugiperda and/or flooding. These data indicate that the observed increase in maysins caused by the combinatorial stress of flooding and S. frugiperda herbivory is not due to increased expression of Sm1 and Sm2.

Due to the increased production of the herbivore-induced volatiles linalool and TMTT in response to the combined stress of flooding and S. frugiperda herbivory, the relative expression of the gene coding for the volatile biosynthesis enzyme terpene synthase 2 (Tps2, GRMZM2G046615) was assessed (Figure 8). TPS2 produces linalool, nerolidol, and geranyllinalool. Linalool can then be converted into DMNT and geranyllinalool into TMTT by a pair of cytochrome p450 enzymes (Richter et al., 2016). Exposure to either flooding or S. frugiperda herbivory alone led to significant increases in the level of Tps2 expression, and the combined effect of flooding and S. frugiperda herbivory produced an additive effect on Tps2 expression. This expression pattern mirrors the production of linalool and indicates that the observed changes in linalool are likely due to changes in expression of Tps2.

4 | DISCUSSION

In this study, the impact of flooding on the responses of maize to biotic attack, specifically S. frugiperda infestation, is examined. The combined stress of flooding plus biotic attack leads to a dramatic increase in accumulation of the plant hormone salicylic acid that is not observed in response to either of the stressors in isolation. The elevated salicylic acid production contributes to the development of increased resistance to S. frugiperda infestation in flooded plants.

There are many examples of plant hormones playing contrasting roles in defence against different pests. The broad consensus advocates that salicylic acid coordinates the defences against many piercing-sucking insects and biotrophic pathogens, whereas jasmonic acid is the major hormone that controls responses to chewing herbivores and necrotrophic fungi (Bari & Jones, 2009; Howe, 2004). In maize, jasmonic acid is a major regulator of defences against S. frugiperda, whereas salicylic acid is not induced in response to infestation in healthy plants. Salicylic acid does, however, accumulate in maize in response to abiotic stresses such as salt stress (Szalai & Janda, 2009).

In many species, negative crosstalk occurs between the jasmonic acid and salicylic acid regulated pathways (Thaler, Humphrey, & Whiteman, 2012). This antagonism, however, seems unlikely to occur in maize, as pretreatment of maize roots with low doses of salicylic acid before leaf elicitation with insect oral secretions leads to increased production of jasmonic acid and higher emissions of linalool, DMNT, and indole but not sesquiterpenes (Engelberth, Viswanathan, & Engelberth, 2011). Interestingly, in our study, there is no obvious effect of the elevated salicylic acid induced during combinatorial stresses involving flooding on insect-induced jasmonic acid production, suggesting that the observed changes in resistance are not due to salicylic acid boosting jasmonic acid production.

The lack of impact of flooding on herbivory-induced jasmonic acid production has also been observed in the roots of rice (Oryza sativa) in response to two root-feeding insects: cucumber beetle (Diabrotica balteata) and rice water weevil (Lissorhoptrus oryzophilus), though in this case, there was no induction of salicylic acid and the effect of flooding on insect resistance was not determined (Lu et al., 2015). Flooding also did not impact the production of herbivory induced jasmonic acid, salicylic acid, or the growth of Spodoptera exigua (beet armyworm) on Solanum dulcamara plants (Nguyen, D'Agostino, et al., 2016). It is therefore likely that the impact of flooding on insect resistance in plants depends upon the plant studied and possibly even the specific plant herbivore combination.

The elevated resistance to S. frugiperda observed in maize plants undergoing flooding stress appears to be due to several factors. The primary of which is the elevated production of salicylic acid, as the loss of salicylic acid signalling in the npr1 mutant significantly attenuates the flood-induced resistance. Another possibly contributing factor is the increased production of anti-insect maysins that are induced by the combined stress of herbivory and flooding. Maysins have been shown to have a negative impact on larval weight gain for several maize pests including Helicoverpa zea (corn earworm) and Helicoverpa armigera (old world corn ear worm; Guo et al., 1999; Rector, Liang, & Guo, 2003; Waiss et al., 1979).

The lack of observed differences in expression of the maysin biosynthesis genes Sm1 and Sm2, coupled with elevated production of the phenylpropanoid precursors of maysins, suggests that the increased accumulation of maysins may be a result of increased precursor availability due to phenylpropanoid pathway remodelling rather than maysin-specific pathway upregulation. The “normal” maysin production in the npr1 mutant supports the notion that salicylic acid is not involved in the increased production of maysins even though it is likely involved in regulating the production of certain phenylpropanoid metabolites including benzoic acid and cinnamic acid. Furthermore, one can extrapolate from this, that, despite their increased production, maysins are not the determining factor of flood-induced S. frugiperda resistance in maize. As a side note, the observed decrease in maysins during infestation in nonflooded plants suggests that S. frugiperda may actively suppress the production of maysins in leaf tissue.

Increased precursor availability due to phenylpropanoid pathway remodelling may also be responsible for the elevated production of the phenolic volatiles, benzyl acetate, and phenethyl acetate. These compounds are likely produced by BAHD acyltransferases (Chedgy, Kollner, & Constabel, 2015; D'Auria, Chen, & Pichersky, 2002; D'Auria, Pichersky, Schaub, Hansel, & Gershenzon, 2007), possibly by the same enzyme that produces the green leaf volatile (Z)-3-hexen-1-yl acetate. However, the specific enzymes responsible for producing these volatiles are not currently known in maize.

Other observed metabolic changes from this combinatorial stress include decreased peroxidase and polyphenol oxidase activities. Cold, heat, and drought have been shown to increase the production of phenolics and decrease polyphenol oxidase and peroxidase activities in various plant species, a combination that is hypothesized to improve the antioxidant status of the plant (Rivero et al., 2001; Sofo, Dichio, Xiloyannis, & Masia, 2005). These changes could also potentially impact herbivore resistance. The metabolic changes observed in our study indicate that both synergistic and additive effects exist in maize to the responses of flooding and herbivory and reveal a hitherto unsuspected role for salicylic acid in defence against chewing herbivores in maize. As little to nothing is known about the function of salicylic acid in regulating anti-herbivore defences in maize, future studies utilizing this stress combination have the potential to reveal hidden anti-herbivore defence pathways that could be exploited to increase pest resistance in maize.

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AUTHOR CONTRIBUTIONS

A.B. conceived the study and designed the experiments. A.B. and S.M. performed the insect-related bioassays and A.B., S.C., S.M., and G. B the metabolite/enzyme activity assays; C.H. performed the gene expression analysis and identified and characterized the npr1 mutant; S.S. performed the total and cell wall bound phenolics analysis, and C.R. developed the maysin analysis protocol. A.B. wrote the manuscript with contributions from all authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Salicylic, Benzoic and Cinnamic acid levels in flooded maize. Non treated (NT) and flooded (FL) maize plants were infested with S. frugiperda (FAW) or Not-infested (NI) and salicylic acid (A), benzoic acid (B) and cinnamic acid (C) levels measured at 2 days after infestation, n = 4, bars = mean ± SEM. Different letters indicate significantly different treatments, $P \leq 0.05$, by ANOVA.

Figure S2. Phylogeny and gene structure of the maize NPR1 ortholog. A) Phylogenetic analysis of the NPR1-containing gene family for maize, rice, and Arabidopsis shows ZmNPR1 is orthologous to AtNPR1. TheNPR1-containing family included NPR1 through NPR4 and BOP1 and BOP2 from Arabidopsis, Bric-a-Brac, Tamrack, Broad Complex BTB (BTBA) genes 1 through 6 from rice, and seven unannotated maize genes. The maize genes were assigned names based on their positions in the tree. The putative ZmNPR1 is denoted with an asterisk. The neighbor-joining tree was constructed using MUSCLE alignments of predicted protein sequences based on primary transcripts of each gene. Bootstrap values for relevant branches are indicated. B) The UniformMu insertion alallele is caused by a Mutator transposable element within the coding sequence of the first exon for ZmNPR1. The gene diagram shows exons in black, untranslated regions as open boxes, and introns as thin lines.

Table S1. Sequences of primers used in study.

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