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OPDA regulates maize defense against aphids

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12-Oxo-phytodienoic acid acts as a regulator of maize defense against corn leaf aphid

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One-sentence summary

12-Oxo-phytodienoic acid promotes enhanced callose accumulation and heightened maize resistance against aphids.

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ABSTRACT

The corn leaf aphid (CLA; *Rhopalosiphum maidis*) is a phloem sap-sucking insect that attacks many cereal crops, including maize (*Zea mays*). We previously showed that the maize inbred line Mp708, which was developed by classical plant breeding, provides enhanced resistance to CLA. Here, using electrophysiological monitoring of aphid feeding behavior, we demonstrate that Mp708 provides phloem-mediated resistance to CLA. Furthermore, feeding by CLA on Mp708 plants enhanced callose deposition, a potential defense mechanism utilized by plants to limit aphid feeding and subsequent colonization. In maize, benzoxazinoids (BX) or BX-derived metabolites contribute to enhanced callose deposition by providing heightened resistance to CLA. However, BX and BX-derived metabolites were not significantly altered in CLA-infested Mp708 plants, indicating BX-independent defense against CLA. Evidence presented here suggests that the constitutively higher levels of 12-oxo-phytodienoic acid (OPDA) in Mp708 plants contributed to enhanced callose accumulation and heightened CLA resistance. OPDA enhanced the expression of ethylene biosynthesis and receptor genes, and the synergistic interactions of OPDA and CLA feeding significantly induced the expression of the transcripts encoding Maize insect resistance1-Cysteine Protease (Mir1-CP), a key defensive protein against insect pests, in Mp708 plants. Furthermore, exogenous application of OPDA on maize jasmonic acid (JA)-deficient plants caused enhanced callose accumulation and heightened resistance to CLA, suggesting that the OPDA-mediated resistance to CLA is independent of the JA pathway. We further demonstrate that the signaling function of OPDA, rather than a direct toxic effect, contributes to enhanced CLA resistance in Mp708.

INTRODUCTION

Despite being a major cereal crop grown worldwide for food, feed, and fuel, maize (*Zea mays*) is attacked by a plethora of insect pests. Among these insect pests, corn leaf aphids (CLA; *Rhopalosiphum maidis*) constitute the largest group of phloem-feeding insects that limit maize productivity (Bing and Guthrie, 1991; Meihls et al., 2012). In addition to removing nutrients from phloem sap and altering source-sink patterns, which negatively affects plant productivity, CLA also is a vector for several plant viral diseases (Thongmeearkom et al., 1976; Carena and Glogoza, 2004; So et al., 2010). Furthermore, heavy CLA infestations on maize result in wilting, curling, and discoloration of leaves. Digestive waste products of CLA (e.g., honeydew), which are deposited on the maize leaf surface, promote mold growth and reduce photosynthetic efficiency, thereby accentuating damage (Carena and Glogoza, 2004).

Phloem-sap-sucking insects, such as CLA, utilize their long slender stylets to penetrate plant tissues and consume nutrients in the sap. Salivary secretions released by aphids enable them to successfully colonize host plants and circumvent activation of plant defenses. Aphids, while feeding on the host plants, inject salivary secretions that potentially interfere with sealing of sieve elements. Aphids release two types of salivary secretions: gelling or sheath saliva and watery saliva. Sheath saliva rapidly sets and seals the wound imposed by stylet penetration and impedes the release of host factors that contribute to the plugging of phloem sieve plates upon aphid stylet insertion (Miles, 1999; Will and Vilcinskas, 2015). On the other hand, watery saliva is secreted continuously during feeding and interacts with phloem proteins, thereby blocking their coagulation. Moreover, the watery saliva contains several enzymes that inhibit phloem sealing and callose deposition, thereby allowing aphids to feed continuously from a single sieve element (Miles, 1999). In addition, several studies have shown that some of these aphid salivary components function as effectors that modulate the plant defense responses (Mutti et al., 2008; Atamian et al., 2013; Chaudhary et al., 2014; Elzinga et al., 2014; Kettles and Kaloshian, 2016; Mugford et al., 2016; Rodriguez et al., 2017). In response, plants use an extensive array of defenses to prevent aphid feeding and colonization. Callose deposition, one of the defense mechanisms utilized by plants, contributes to sieve element occlusion and, thus, control of infestation by phloem-feeding insects (Will and van Bel, 2006). For example, callose deposition in the sieve elements is associated with resistance in rice (*Oryza sativa*) against brown

planthopper (*Nilaparvata lugens*) (Hao et al., 2008). In addition, Arabidopsis (*Arabidopsis thaliana*) responds to silverleaf whitefly (*Bemisia tabaci*) and green peach aphid (*Myzus persicae*) infestations by enhancing callose deposition and expression of the callose synthase encoding genes (Kempema et al., 2007; Casteel et al., 2014; Mondal et al., 2018).

Benzoxazinoids (BX), a class of secondary metabolites, contribute to maize defense against CLA (Ahmad et al., 2011). The CLA population was significantly higher on BX-deficient maize plants. Furthermore, the enhanced CLA numbers on BX-deficient maize lines correlated with the reduced accumulation of callose (Ahmad et al., 2011). 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), an intermediate compound in the BX pathway, acts as a signaling molecule in regulating CLA feeding-induced callose accumulation in resistant maize lines (Ahmad et al., 2011). Indeed, infiltration of DIMBOA into the maize leaves stimulated callose accumulation. In addition, the parental lines of the maize nested association mapping (NAM) population that had elevated levels of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-Glc), the precursor for 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), were more resistant to CLA by enhancing callose accumulation (Meihls et al., 2013). These studies confirm that the BX or BX-derived metabolites are involved in enhancing callose accumulation, thus providing elevated maize resistance to CLA.

We have previously shown that the maize inbred line Mp708 has enhanced defense against CLA (Louis et al., 2015). CLA feeding on Mp708 plants rapidly induced the accumulation of the transcripts encoding Maize insect resistance1-Cysteine Protease (Mir1-CP) defensive protein. Mir1-CP is localized to the vascular tissues, and feeding trial bioassays have confirmed that the recombinant Mir1-CP adversely influences CLA fecundity (Lopez et al., 2007; Louis et al., 2015). Furthermore, aboveground feeding by CLA rapidly sends as yet unidentified signals to the roots that trigger belowground accumulation of *mir1* (Louis et al., 2015; Varsani et al., 2016). In support of a role for an aboveground-belowground signaling mechanism, CLA-feeding-induced *mir1* expression provided enhanced resistance to subsequent belowground feeding of western corn rootworm (*Diabrotica virgifera virgifera*) (Varsani et al., 2016). Root removal prior to CLA infestation significantly affected the accumulation of *mir1* transcripts in the aboveground whorl region of Mp708 plants. These results, in conjunction with

187 the observation that roots act as a site for Mir1-CP synthesis in response to foliar CLA feeding,
188 suggest that the presence of Mir1-CP in the vascular tissues contributes to enhanced resistance to
189 CLA.
190
191 In addition to defensive proteins, phytohormones, including jasmonic acid (JA), salicylic
192 acid (SA), and ethylene (ET), interactively modulate plant defenses against insect herbivory
193 (Howe and Jander, 2008; Erb et al., 2012; Louis and Shah, 2013). For example, it has been
194 shown that elevated levels of SA due to loss of *FATTY ACID DESATURASE7* (*FAD7*) activity in
195 tomato (*Solanum lycopersicum*) resulted in hyperresistance against potato aphids (*Macrosiphum*
196 *euphorbiae*) (Avila et al., 2012). In addition to SA, JA plays a critical role in providing resistance
197 against aphids. In sorghum (*Sorghum bicolor*), methyl jasmonate treatment of seedlings resulted
198 in fewer numbers of greenbug aphids (*Schizaphis graminum*) compared to the untreated control
199 plants, suggesting the significance of JA-pathway-mediated defense in sorghum against aphids
200 (Zhu-Salzman et al., 2004). In addition, it has been shown that *AKR* (*Acyrthosiphon kondoi*
201 resistance)-mediated resistance against blue green aphids (*Acyrthosiphon kondoi*) in *Medicago*
202 *truncatula* and Arabidopsis resistance against cabbage aphids (*Brevicoryne brassicae*) require
203 the JA pathway (Gao et al., 2007; Kuśnierczyk et al., 2011). ET, primarily considered to be
204 synergistic with JA, also was shown to be induced by aphid infestation in resistant varieties of
205 tomato and melon (Anstead et al., 2010). Recent studies on maize-CLA interaction suggested a
206 potential role of SA-JA antagonism and ET in modulating defenses against aphids (Louis et al.,
207 2015; Tzin et al., 2015). The maize Mp708 genotype has constitutively elevated levels of JA and
208 12-oxo-phytodienoic acid (OPDA) (Shivaji et al., 2010). Previously, it was suggested that JA
209 acts upstream of ET in activating *mir1*-mediated defenses in maize against chewing insects
210 (Ankala et al., 2009). However, JA was not a critical component in the *mir1*-determined
211 enhanced resistance to CLA (Louis et al., 2015). Instead, the enhanced *mir1*-determined
212 resistance to CLA in the Mp708 genotype depended only on the ET pathway (Louis et al., 2015).
213
214 OPDA, an intermediate in the JA biosynthesis pathway, can contribute to plant defense
215 against insect pests. For example, OPDA stimulates enhanced resistance in rice and wheat
216 (*Triticum aestivum*) against brown planthopper and Hessian fly (*Mayetiola destructor*),
217 respectively (Guo et al., 2014; Cheng et al., 2018). Similarly, Arabidopsis *opr3* plants, which are

deficient in JA but accumulate elevated levels of OPDA, were shown to have enhanced resistance to the dipteran insect *Bradysia impatiens* (Stintzi et al., 2001). By contrast, cabbage loopers (*Trichoplusia ni*) reared on the Arabidopsis *opr3* plants had significantly higher weight than the wild-type plant, suggesting that OPDA may not be a critical component in providing resistance to chewing herbivores (Chehab et al., 2011). As mentioned before, insects release salivary secretions while feeding, which could potentially activate wound-induced signaling molecules, such as OPDA, and trigger the downstream defenses in plants (Park et al., 2013; Bosch et al., 2014a, 2014b; Guo et al., 2014; López-Galiano et al., 2017). More recent studies have suggested that oxylipins, a large family of oxidized lipids including OPDA, are involved in enhancing callose accumulation in host plants to limit pathogen infection (Marcos et al., 2015; Scalschi et al., 2015), which is also a potential defense mechanism utilized by plants to disrupt aphid colonization.

In this study, we investigated whether the constitutively elevated levels of OPDA in the Mp708 genotype are critical for the *mir1*-mediated defense against CLA. We demonstrate that the Mp708 genotype provides enhanced resistance to CLA by promoting callose accumulation, independent of the BX pathway. Our data suggest that OPDA is involved in activating callose formation and enhanced resistance to CLA in Mp708 plants. OPDA application enhances the expression of ET biosynthesis and receptor genes, which act as a central node in regulating *mir1* expression to different feeding guilds of insect herbivores (Louis et al., 2015). We further show that the OPDA-mediated enhanced callose accumulation and resistance to CLA is independent of the JA pathway. Our results also suggest that the signaling function of OPDA (Taki et al., 2005; Böttcher and Pollman, 2009), not the direct toxic effect, contributes to heightened resistance to CLA in Mp708 plants.

RESULTS

The Maize Inbred Line Mp708 Promotes Phloem-Based Resistance to CLA

Previously, we showed that Mp708 promotes enhanced resistance to CLA (Louis et al., 2015). We utilized the electrical penetration graph (EPG) technique to monitor and quantify the different CLA feeding patterns on resistant (Mp708) and susceptible (Tx601 and B73) maize

genotypes. The different waveform patterns quantified from the EPG experiments include (1) total duration of the pathway phase (PP) that includes both the inter- and/or intracellular aphid stylet routes during the brief sampling of cells; (2) total duration of nonprobing phase (NP) that includes relatively no aphid stylet movement or activity on the plant tissues; (3) time to reach first sieve element phase (f-SEP); (4) total duration of sieve element phase (SEP) or phloem phase when the aphid stylets are in the phloem/sieve element and actively ingest nutrients; and (5) total duration of xylem phase (XP) when the aphid inserts its stylets into the xylem and feeds on the xylem sap. There were no significant differences ($P > 0.05$; Kruskal-Wallis test) in the PP, NP, f-SEP, and XP waveform patterns measured for CLA feeding behavior on the resistant Mp708 and susceptible Tx601 genotypes (Fig. 1; Table 1). However, CLA spent significantly less time in the sieve elements of the resistant maize genotype Mp708 compared to Tx601 plants, suggesting that Mp708's resistance to CLA is phloem-localized (Fig. 1; Table 1). Figure 1B shows the representative EPG waveform patterns produced by CLA feeding on resistant Mp708 and susceptible Tx601 genotypes. Similarly, comparison of CLA feeding behavioral activities between Mp708 and B73, a reference maize line that supports CLA numbers comparable to the Tx601 genotype (Louis et al., 2015), revealed that CLA spent significantly less time feeding from the sieve elements of Mp708 plants (Supplemental Fig. S1, A and B). These data suggest that Mp708 promotes phloem-based resistance to CLA and restricts the sustained feeding of CLA from the sieve elements.

CLA Infestation Enhanced Callose Deposition in Mp708 Plants

Callose deposition is an important plant defense mechanism that contributes to phloem occlusion and thereby controls the infestation of phloem-feeding insects (Will and van Bel, 2006; Hao et al., 2008; Mondal et al., 2018). Since Mp708 plants restrict CLA ability to continuously feed from the sieve elements, we monitored the temporal accumulation of callose in Mp708 and Tx601 maize genotypes before and after CLA infestation. Interestingly, Mp708 plants had constitutively higher callose spots compared to Tx601 genotypes (Fig. 2A). In addition, Mp708 plants had significantly higher callose accumulation through 24 h of CLA infestation compared to the Tx601 genotype (Fig. 2A). We also monitored the expression of *Tie-dyed2* (*Tdy2*), a gene highly expressed in the vascular tissues and involved in the synthesis of callose in maize (Slewinski et al., 2012), to investigate whether the enhanced callose accumulation in resistant

maize plants correlates with the higher expression of callose synthase gene. Although *Tdy2* expression was not significantly different between Mp708 and Tx601 plants before CLA infestation, CLA feeding for 24 h significantly increased the expression of *Tdy2* in Mp708 plants compared to Tx601 plants (Fig. 2B). These findings, coupled with the EPG experiments in which we observed reduced aphid feeding from the sieve elements of Mp708 plants, suggest that enhanced callose deposition in the resistant maize genotype restricts sustained aphid feeding.

BX or BX-Derived Metabolites Are Not Significantly Altered in CLA-Infested Mp708 Plants

Indole-derived BX act as key defensive secondary metabolites against insect attack in maize (Meihls et al., 2012). DIMBOA-Glc and 2-hydroxy-4,7- dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) constitute the most abundant BX in maize (Frey et al., 2009; Meihls et al., 2013). Furthermore, DIMBOA, a breakdown product of DIMBOA-Glc, was sufficient to trigger callose deposition in maize (Ahmad et al., 2011; Meihls et al., 2012, 2013; Betsiashvili et al., 2015). To test the possible role of DIMBOA and breakdown products of DIMBOA in enhanced callose accumulation in Mp708 plants, we monitored the temporal accumulation of BX-derived metabolites before and after CLA infestation. As shown in Figure 3A, comparison of Tx601 and Mp708 plants revealed that DIMBOA-Glc concentration was not changed at early time points of CLA feeding but was significantly increased in the susceptible Tx601 plants after 24 h of CLA feeding. DIMBOA-Glc concentration in the resistant Mp708 genotype was not significantly altered over the 24-h period of CLA feeding (Fig. 3A). HDMBOA-Glc and DIMBOA abundance were comparable in the Tx601 and Mp708 plants before and after CLA infestation (Fig. 3, B and C). Similarly, two downstream metabolites of DIMBOA-Glc, 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one glucoside (DIM2BOA-Glc) and 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one (DIM2BOA) that can also contribute to CLA resistance (Handrick et al., 2016), were not significantly altered before and after CLA infestation in the Tx601 and Mp708 plants (Fig. 3, D and E). Furthermore, we monitored the expression of several genes involved in the BX biosynthesis (Tzin et al., 2017), before and after CLA infestation. Although Mp708 plants had constitutively elevated expression of *BX1* compared to Tx601 plants, CLA feeding for 24 h suppressed the expression of *BX1* in Mp708 plants and was comparable to the susceptible Tx601 plants (Supplemental Fig. S2A). Additionally, expression

of *BX7* and *BX11* genes was comparable in Tx601 and Mp708 plants before and after CLA infestation for 24 h (Supplemental Fig. S2, B and C). Although there was higher expression of *BX13* in CLA uninfested Tx601 and Mp708 plants, CLA feeding for 24 h significantly decreased *BX13* transcript expression on both maize genotypes and was comparable in Tx601 and Mp708 plants (Supplemental Fig. S2D). Collectively, these data suggest that BX or BX-derived metabolites are not major contributors to the Mp708 resistance to CLA, and defense signals other than DIMBOA and/or BX-derived metabolites may be involved in activating aphid-induced callose formation in Mp708 plants.

OPDA Promotes Enhanced Callose Deposition and Heightened Resistance to CLA in Mp708 Plants

As found previously (Shivaji et al., 2010), resistant Mp708 plants had constitutively higher levels of OPDA and JA compared to susceptible Tx601 plants. However, higher levels of JA were not critical for providing defense against CLA in Mp708 plants (Louis et al., 2015). OPDA, a precursor for JA biosynthesis, is also involved in activating plant defenses not related to the JA pathway (Taki et al., 2005; Böttcher and Pollman, 2009). Furthermore, it has been shown that OPDA enhances plant defenses by inducing callose accumulation (Scalschi et al., 2015). To investigate whether the elevated levels of OPDA contribute to callose accumulation in maize plants, we pretreated Tx601 and Mp708 genotypes with OPDA for 24 h. Our results indicate that the OPDA pretreatment (+ OPDA) alone significantly increased callose accumulation and expression of *Tdy2* in Mp708 plants (+ OPDA) compared with Mp708 control plants (- OPDA) (Fig. 4A; Supplemental Fig. S3A). In contrast, exogenous application of OPDA did not elicit a significant increase in the callose accumulation and *Tdy2* transcript levels in Tx601 plants (+ OPDA) compared to Tx601 control plants (- OPDA) (Fig. 4A; Supplemental Fig. S3B). When compared with Mp708 plants infested with CLA, exogenous application of OPDA and subsequent feeding by CLA significantly increased the callose deposition in Mp708 plants (Fig. 4A). However, this was not the case with Tx601 plants, where we observed no significant difference in the callose accumulation after CLA infestation with and without exogenous application of OPDA (Fig. 4A). These results indicate that the OPDA and CLA feeding interact synergistically to promote enhanced callose deposition in Mp708 plants.

To test whether exogenous application of OPDA contributes to enhanced resistance to CLA, we pretreated maize plants with OPDA (50 µM) 24 h prior to aphid release. OPDA pretreatment contributed to enhanced resistance in Mp708 plants compared with Mp708 control plants (- OPDA) (Fig. 4B). However, OPDA pretreatment of Tx601 plants did not adversely affect the CLA population compared with Tx601 control plants (- OPDA) (Fig. 4B). These results further confirmed the positive influence of OPDA on callose deposition and heightened resistance to CLA in Mp708 plants.

To confirm the observed role of OPDA in promoting enhanced callose deposition and heightened resistance to CLA in Mp708 plants, we pretreated the plants with either 2-deoxy-D-glucose (DDG), an inhibitor of callose synthesis in plants (Jakab et al., 2001; Hamiduzzaman et al., 2005), OPDA, or coapplied OPDA and DDG 24 h prior to CLA infestation. DDG application on Mp708 leaves suppressed the expression of callose synthase *Tdy2* transcript abundance (Supplemental Fig. S4). Mp708 plants treated with DDG prior to CLA infestation supported higher numbers of aphids in a no-choice bioassay compared to Mp708 control plants (Fig. 5). As expected, OPDA-treated Mp708 plants provided enhanced resistance to CLA compared to Mp708 control plants. However, coapplication of OPDA and DDG did not restore the resistance phenotype of Mp708 plants against CLA. The aphid numbers were comparable to Mp708 plants that were pretreated with DDG alone (Fig. 5), indicating that OPDA acts upstream of callose accumulation and may have a direct role in the regulation of callose accumulation in Mp708 plants. In contrast, no differences in CLA numbers were observed between the control and DDG pretreated Tx601 plants (Supplemental Fig. S5). The aphid bioassay data, which indicate that OPDA promotes heightened resistance to CLA (Fig. 5), was further supported by EPG studies where we monitored the feeding behavior of CLA on Mp708 plants after pretreatment with either DDG, OPDA, or coapplication with OPDA and DDG 24 h prior to CLA infestation. The duration of time spent by CLA in the sieve element phase (SEP) was considerably shorter in OPDA-pretreated Mp708 plants compared to Mp708 control plants (Supplemental Table S1), indicating that OPDA-promoted callose accumulation deters CLA feeding from sieve elements. However, the aphids were able to overcome this feeding block when the Mp708 plants were pretreated with DDG or coapplied with OPDA and DDG (Supplemental Table S1). In addition, we observed a corresponding reduction in the duration of the pathway phase, during which the

aphids puncture the different plant cells to locate sieve elements, when the plants were pretreated with DDG or coapplied with OPDA and DDG compared to control Mp708 plants (Supplemental Table S1). These results suggest that the OPDA-mediated callose accumulation deters aphid feeding from sieve elements and subsequently promotes enhanced resistance to CLA in the Mp708 genotype.

OPDA Application Enhances CLA-Feeding-Induced *mir1* and Ethylene Biosynthesis and Receptor Genes in Mp708 Plants

To further examine the role of OPDA in activating other defense responses in Mp708 plants, including the ET pathway and its interaction with the *mir1* defensive gene (Louis et al., 2015), we monitored the expression of maize ethylene biosynthesis and receptor genes (Young et al., 2004; Yamauchi et al., 2016) and *mir1* gene activation. Pretreatment of Mp708 plants with OPDA significantly induced the expression of maize ethylene biosynthesis (*Aminocyclopropane-1-Carboxylic acid Synthase 2* [*ACS2*], *ACS6,* and *ACC Oxidase 15* [*ACO15*]) and receptor (*Ethylene Response Sensor 14* [*ERS14*]) genes (Fig. 6, A-D). However, the same treatment did not significantly alter the response of ethylene biosynthesis gene in Tx601 plants (Supplemental Fig. S3C). In addition, exogenous OPDA application and subsequent feeding by CLA significantly increased the expression of maize ethylene biosynthesis and receptor genes in Mp708 plants compared to Mp708 plants infested with CLA (Fig. 6, A-D). Analysis of *mir1* expression revealed that OPDA treatment alone did not enhance the *mir1* transcript accumulation. However, synergistic interactions of OPDA and CLA feeding significantly increased *mir1* transcript accumulation compared to CLA feeding alone on Mp708 plants (Fig. 6E). These results suggest that OPDA activates the ET pathway and potentially regulates *mir1* transcript accumulation in Mp708 plants.

Exogenous Application of Methyl Jasmonate Did Not Significantly Increase the Callose Deposition in Mp708 Plants

Previously, we showed that the exogenous application of Mp708 plants with methyl jasmonate (MeJA) did not significantly alter the CLA population size compared to Mp708 control plants (Louis et al., 2015). Here, we pretreated the Mp708 plants with MeJA for 24 h and monitored the accumulation of callose in Mp708 plants before and after MeJA treatment. Our results indicate

that the MeJA pretreatment alone did not significantly increase the number of callose spots compared to untreated Mp708 control plants (Fig. 7). Furthermore, there was no significant difference in the number of callose spots when comparing Mp708 plants infested with CLA and exogenous application of MeJA followed by CLA feeding (Fig. 7). These findings indicate that the JA is not required for enhanced callose accumulation in Mp708 plants.

OPDA-Mediated Resistance to CLA Is Independent of the JA Pathway

To determine whether the OPDA-mediated resistance to CLA in maize can occur independently of the JA pathway, we used a maize JA-deficient mutant line in B73 background, which is disrupted in two *12-Oxo-Phytodienoic acid Reductase* (*OPR7* and *OPR8*) genes (Yan at al., 2012). Wound-induced OPDA levels in *opr7 opr8* double mutants were comparable to wild-type plants, whereas JA induction was not detectable in *opr7 opr8*, indicating that *OPR7* and *OPR8* function in the conversion of OPDA to JA (Yan et al., 2012). Aphid no-choice bioassays showed comparable numbers of CLA on the wild-type and *opr7 opr8* controls plants after 4 days post infestation, whereas CLA counts were significantly lower on the *opr7 opr8* plants that were pretreated with OPDA for 24 h (Fig. 8A). We further determined whether the exogenous application of OPDA could also increase callose deposition in the *opr7 opr8* plants. Our results indicate that the OPDA pretreatment alone did not significantly increase the number of callose spots in *opr7 opr8* plants compared with the wild-type plants (Fig. 8B). However, exogenous application of OPDA and subsequent feeding by CLA significantly increased the callose spots in *opr7 opr8* plants compared with the *opr7 opr8* control plants and wild-type plants with or without OPDA treatment (Fig. 8B). These results indicate that JA is not required for CLA resistance and that the OPDA-mediated resistance to CLA is independent of the JA pathway.

In comparison to Tx601 plants, Mp708 plants had constitutively elevated levels of OPDA, JA, and JA-related defenses (Shivaji et al., 2010). We further quantified the levels of OPDA, JA, and JA-Ile before and after treating the Mp708 plants with OPDA. As shown previously (Shivaji et al., 2010), Mp708 plants had constitutively higher levels of OPDA, JA, and JA-Ile compared to Tx601 plants (Supplemental Fig. S6). Exogenous application of OPDA on Mp708 plants did not significantly increase the levels of JA and JA-Ile compared to Mp708 control plants. In fact, OPDA treatment of Mp708 plants significantly reduced the levels of JA

and JA-Ile compared to Mp708 control plants (Supplemental Fig. S6). These results further confirm a JA-independent role of OPDA in regulating defense against CLA.

OPDA Does Not Have a Direct Negative Impact on CLA Growth and Fecundity

To determine whether OPDA has a direct negative effect on CLA growth and fecundity, we performed a feeding trial bioassay in which CLA was reared on an artificial diet containing 50 or 200 µM OPDA for 4 days. Our aphid feeding assays confirmed that OPDA in the artificial diet did not negatively affect the CLA growth and fecundity compared to CLA reared on diet alone and the diet mixed with DMSO, the solvent used to make the OPDA stock solution (Fig. 9). This result suggests that the elevated level of OPDA in Mp708 is unlikely to directly contribute to Mp708 resistance to CLA. Instead, OPDA-induced activation of downstream defenses likely contributes to the resistant phenotype of Mp708 against CLA.

DISCUSSION

Besides acting as a precursor for JA biosynthesis, OPDA can activate downstream signaling mechanisms and promote enhanced callose accumulation (Taki et al., 2005; Böttcher and Pollman, 2009; Scalschi et al., 2015; Wasternack and Hause, 2016; Wasternack and Strnad, 2016; Monte et al., 2018). Mp708 plants had constitutively elevated levels of both JA and OPDA (Shivaji et al., 2010). However, previously, we suggested that Mp708 resistance against CLA is independent of the JA pathway (Louis et al., 2015). Here, we monitored whether elevated levels of OPDA can contribute to maize defense against CLA. Exogenous OPDA application promoted increased callose deposition and heightened CLA resistance in Mp708 plants (Fig. 4). Furthermore, OPDA pretreatment and CLA feeding triggered the ET pathway and *mir1* transcript accumulation (Fig. 6), suggesting that OPDA acts as a signaling molecule to trigger downstream defense responses.

Several studies have suggested an important role for oxylipins in modulating plant defenses against aphids (Smith et al., 2010; Nalam et al., 2012; Avila et al., 2013; Guo et al., 2014). For example, the oxylipin 9-hydroxyoctadecadienoic acid (9-HOD) was involved in promoting aphid colonization and fecundity on Arabidopsis (Nalam et al., 2012). In contrast, α-

dioxygenases (α-DOX1)-derived oxylipins contributed to aphid resistance in both Arabidopsis and tomato (Avila et al., 2013). Similarly, OPDA was involved in activating plant defense responses to aphids in both wheat and radish (*Raphanus* sp.) (Smith et al., 2010; Guo et al., 2014). Several diverse lipids, including oxylipins, have been identified in phloem sap as well (Madey et al., 2002; Harmel et al., 2007; Benning et al., 2012). However, it is not known whether the oxylipin-based defenses against aphids are exerted within or outside of the phloem sap. Our results demonstrate that it is highly unlikely that OPDA has a direct negative effect on aphid growth and fecundity because artificial diet assays confirmed that OPDA does not limit CLA growth and fecundity (Fig. 9). Alternatively, aphids may have the ability to convert the ingested OPDA into a less toxic form. In fact, it has been shown that some chewing insects isomerize OPDA into a less toxic form that is excreted in the frass (Dabrowska et al., 2009). Although the exact mechanisms by which aphids sequester and/or avoid the effect of OPDA on aphid physiology is unknown, our results suggest that the signaling function of OPDA is likely responsible for providing enhanced defense against CLA in Mp708 plants.

OPDA treatment triggers the expression of ET biosynthesis and receptor genes (Fig. 6, A-D) that are involved in the production of ET in maize (Young et al., 2004; Yamauchi et al., 2016). In a previous study, the ET signaling pathway was correlated with promoting pathogen-induced callose deposition in Arabidopsis, thereby providing enhanced resistance (Clay et al., 2009). However, it was also reported that Arabidopsis plants can induce pathogen-triggered callose accumulation in a glucosinolate-independent manner (Frerigmann et al., 2016). Whatever the precise mechanisms involved, our data suggest that the OPDA-triggered ET pathway and its interaction with the *mir1* defensive gene (Fig. 6; Louis et al., 2015) contribute to enhanced resistance to CLA potentially by enhancing callose accumulation and limiting the aphid growth. Furthermore, MeJA, which is derived from JA, antagonizes the ET pathway and suppresses pathogen-triggered callose deposition in Arabidopsis (Clay et al., 2009). Similarly, exogenous application of JA on tomato plants was negatively correlated with callose accumulation (Scalschi et al., 2015), further suggesting that JA or MeJA suppresses callose accumulation in plants. Although elevated JA levels in Mp708 were not required for the *mir1*-dependent defense against CLA (Louis et al., 2015), we cannot rule out the possibility that the JA and/or JA-derived compounds also modulate callose deposition in maize. However, this is less likely, considering

497 the fact that we observed no significant differences in the number of callose spots on MeJA-
498 pretreated Mp708 plants compared to control plants (Fig. 7). In addition, OPDA pretreatment
499 followed by CLA feeding on the JA-deficient *opr7 opr8* mutant plants significantly enhanced
500 callose accumulation compared with *opr7 opr8* control plants and wild-type plants (Fig. 8B),
501 pointing to a role of OPDA in defense against CLA that is independent of JA. Surprisingly,
502 OPDA pretreatment did not alter the CLA population size on B73 and Tx601 plants (Figs. 4B
503 and 8A). One possible explanation is that the OPDA conversion to JA is highly stimulated in
504 both B73 and Tx601 plants, which leads to a corresponding increase in JA and/or JA-dependent
505 defenses and simultaneously weakens the OPDA-modulated defense arm that is independent of
506 the JA pathway. Alternatively, unlike Mp708 plants where there is an effective defense protein
507 such as Mir1-CP, both B73 and Tx601 plants may lack effective defensive proteins that could
508 respond to OPDA and induce downstream defense mechanisms (for example, enhanced callose
509 accumulation).
510
511 Callose accumulation that contributes to aphid resistance could occur within and/or
512 outside of the sieve elements (Hao et al., 2008; Du et al., 2009; Mondal et al., 2018). In both
513 scenarios, it severely hinders the aphid's ability to find and feed continuously from the phloem
514 sap. EPG analysis indicated that Mp708's resistance to CLA is phloem-localized. Furthermore, it
515 is apparent from the EPG experiments that CLA took similar amounts of time to reach the first
516 SEP on both Mp708 and Tx601 plants (Fig. 1), indicating that the callose deposited on the sieve
517 elements could potentially play a significant role in hindering CLA ability to feed continuously
518 on resistant maize plants. Furthermore, OPDA-treated Mp708 plants provided enhanced
519 resistance and prevented aphids from sustained feeding from the sieve elements compared to
520 Mp708 control plants (Fig. 5; Supplemental Table S1). Mp708 plants also had constitutively
521 higher numbers of callose spots compared to Tx601 plants. The endogenous OPDA levels were
522 sufficient to promote constitutively higher callose spots in Mp708 plants compared to Tx601
523 plants (Figs. 2A and 4A). However, we observed comparable levels of callose synthase gene
524 expression (*Tdy2*) in both Mp708 and Tx601 genotypes prior to CLA infestation (Fig. 2B). In
525 contrast, CLA feeding for 24 h significantly increased *Tdy2* expression in Mp708 compared to
526 Tx601 plants (Fig. 2B). It is plausible that, since *Tdy2* is highly expressed in the vascular tissues
527 and involved in the synthesis of callose, Mp708 plants initially need to perceive the salivary

signals from CLA to promote enhanced callose accumulation in the sieve elements. Similarly, OPDA treatment alone did not significantly induce the accumulation of *mir1* (Fig. 6E), a gene that is highly expressed in the vascular tissues of Mp708 plants (Lopez et al., 2007), which may also require the interaction of OPDA and CLA salivary signals to promote *mir1*-dependent defense against CLA. Indeed, we previously showed that the CLA-feeding-induced accumulation of defense molecules or factors in the vascular sap of Mp708 plants contributes to enhanced defense against CLA (Louis et al., 2015).

Callose synthesis inhibitor treatment of susceptible Tx601 plants did not affect CLA growth and reproduction (Supplemental Fig. S5), suggesting that OPDA-mediated callose accumulation, and thus defenses, are attenuated in the susceptible maize plants. Alternatively, CLA salivary secretions may cause unplugging of the sieve element occlusions in the susceptible Tx601 plants. The latter is in agreement with our observation that CLA spent a longer time feeding in the sieve elements of susceptible maize plants (Fig. 1; Supplemental Fig. S1). Furthermore, consistent with our observation that the CLA-susceptible plants were unable to mount appropriate defenses, the B73 maize inbred line, which is susceptible to CLA compared to Mp708 plants (Louis et al., 2015), also demonstrated reduced levels of OPDA or OPDA conjugates after CLA infestation (Tzin et al., 2015). Collectively, our data suggest that the elevated levels of OPDA, in conjunction with CLA feeding, trigger the activation of downstream defenses and callose accumulation in the resistant Mp708 plants.

CONCLUSION

In this study, we provide evidence that the signaling function of OPDA, but not JA, promotes phloem-localized resistance to aphids in maize. Our data suggest that OPDA, in addition to acting as a precursor for JA biosynthesis, is also involved in activating callose formation in resistant maize plants. Moreover, our results indicate that OPDA can influence the ET pathway and its interaction with the *mir1* defensive gene to provide heightened resistance to CLA. The identification of OPDA as a key modulator in regulating defense-signaling pathways could be utilized for enhancing maize resistance to phloem-sap-sucking pests.

559

560 **MATERIALS AND METHODS**

561

562 **Aphid Propagation**

563 A CLA colony was reared on barley (*Hordeum vulgare*) plants as described previously (Louis et
564 al., 2015). The barley seeds were obtained from P. Stephen Baenziger, University of Nebraska-
565 Lincoln (UNL). The aphid colonies were grown in a Percival growth chamber with a 14:10
566 (light:dark) photoperiod, 160 μE $m^{-2}s^{-1}$, 23°C, and 50 to 60% relative humidity.

567

568 **Plants and Growth Conditions**

569 Mp708 and Tx601 maize (*Zea mays*) plants were grown in soil mixed with vermiculite and
570 perlite (PRO-MIX BX BIOFUNGICIDE + MYCORRHIZAE, Premier Tech Horticulture) in
571 growth chambers with a 14:10 (light:dark) photoperiod, 160 μE $m^{-2}s^{-1}$, 25°C, and 50 to 60%
572 relative humidity. The *opr7 opr8* mutant line has been described previously (Yan et al., 2012).
573 The *opr7 opr8* plants used in this study were at the BC7 stage in the B73 background. Seeds
574 segregating for the *opr7 opr8* double mutation in a 1:3 ratio were used in this study. Phenotypic
575 differences (lack of anthocyanin pigmentation in brace roots and leaf collar) and PCR-based
576 genotyping were used to identify the *opr7 opr8* homozygous double mutants as described
577 previously (Yan et al., 2012). Since *opr7 opr8* plants are nonviable in nonsterile soil due to
578 *Pythium* spp. infection (Yan et al., 2012), the *opr7 opr8* and the wild-type (B73) control plants
579 were grown in sterile soil. All plants for the experiments were used at the V2-V3 developmental
580 stage (~2 weeks) (Ritchie et al., 1998). These plants were grown in 3.8 cm x 21.0-cm plastic
581 Cone-tainers (Hummert International).

582

583 **Aphid Feeding Behavior Analysis**

584 The EPG technique (Tjallingii, 1988; Walker, 2000; Louis et al., 2012) was used to monitor the
585 CLA feeding behavior on different maize genotypes, as described previously (Pegadaraju et al.,
586 2007). Briefly, a thin gold wire was attached to the dorsum of apterous adult CLA using
587 conductive water-based silver glue. The wired aphid was placed on a plant that was connected to
588 an EPG-recording system using a copper electrode inserted into the soil. The plants and insects
589 were contained in a Faraday cage during EPG recordings to avoid external electrical noise. The

recordings were performed for 8 h under constant light at an ambient room temperature of 22°C. An eight-channel GIGA-8 direct current amplifier (http://www.epgsystems.eu/; W.F. Tjallingii, Wageningen University, Wageningen, The Netherlands) was used for EPG recordings. Plants were randomized to the eight channels for each recording, and at least 12 replicates of individual aphids (one aphid per plant) were obtained for each maize genotype. The different waveforms obtained were analyzed using the EPG analysis software *Stylet*$^{+}$ (http://www.epgsystems.eu/; W.F. Tjallingii, Wageningen University, Wageningen, The Netherlands).

Callose Staining and Quantification

Callose staining and quantification of callose spots were done as described previously (Luna et al., 2011). Briefly, leaves were collected after 24 h of OPDA (50 µM) or MeJA (500 µM) treatment. Control plants were treated with 0.1% DMSO or 0.1% Tween, which were used to dissolve OPDA or MeJA, respectively. Ten adult apterous CLAs were clip-caged on the leaves for CLA-infested plants. Control plants had empty cages. The leaves were placed in 98% ethanol for 48 h to clear the chlorophyll, and once the leaves become transparent, the leaves were placed in 70% ethanol. The leaves were then gently washed three times using distilled water and stained for 3 to 4 h in 150 nM K_2HPO_4 (pH 9.5) containing 0.01% aniline blue (Sigma-Aldrich). The leaves were mounted on slides using 50% glycerol and were examined with an EVOS FL epifluorescence microscope. Callose spots were counted per mm^2 of leaf tissue on the adaxial side of each clip-caged leaf segment using ImageJ (http://imagej.nih.gov/ij/).

Aphid Bioassays

Aphid no-choice bioassays were performed as described previously (Louis et al., 2015).

Artificial Diet Feeding Trial Bioassays

Aphid feeding trial bioassays were carried out using an artificial diet (Meihls et al., 2013) as previously described (Louis et al., 2015). OPDA (50 or 200 µM; Cayman Chemical) dissolved in 0.1% DMSO (Sigma-Aldrich) or aphid diet mixed with 0.1% DMSO was used as the control for artificial diet feeding assays.

RNA Extraction and Reverse Transcription Quantitative PCR (RT-qPCR)

Maize leaf tissues (80-100 mg) were ground using a 2010 Geno/Grinder (SPEX SamplePrep) for 40 seconds at 1,400 strokes min^{-1} under liquid nitrogen conditions. Total RNA was extracted from the homogenized tissue using the Qiagen RNeasy Plant Mini Kit. Extracted total RNA was quantified with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). Complementary DNAs (cDNAs) were synthesized from 1 μg of total RNA using the High Capacity cDNA reverse transcriptase kit (Applied Biosystems). cDNAs were diluted to 1:10 before using them for RT-qPCR. The RT-qPCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Gene-specific primers used for RT-qPCR are listed in Supplemental Table 2. At least three independent biological replicates were used for RT-qPCR, and each biological replicate contained three technical replicates. Primer efficiencies and relative expression levels were calculated as described previously (Pfaffl, 2001).

BX Quantification

Maize plants were infested with 10 adult apterous CLA using clip cages, and at different time points, CLAs were removed from the leaves and tissues were harvested. Leaves were weighed and immediately flash-frozen in liquid nitrogen. Maize BX extraction and quantification were carried out as described previously (Handrick et al., 2016). Three microliters of extraction solvent (30:69.9:0.1 methanol, LC-MS-grade water [Sigma-Aldrich], formic acid; with 0.075 mM 2-benzoxazolinone) was added per milligram of maize tissue. Samples were mixed by vortexing and were incubated on a Labquake Rotisserie Shaker (Thermo Fisher Scientific) at 4°C for 40 min. After centrifugation at 11,000*g* for 10 minutes, 200 μL of the supernatant was filtered using a 0.45-micron filter-bottom plate and centrifugation at 200*g* for 3 min. Samples were analyzed using an Ultimate 3000 UPLC system attached to a 3000 Ultimate diode array detector and a Thermo Q Exactive mass spectrometer (Thermo Fisher Scientific). The samples were separated on a Titan C18 7.5 cm x 2.1 mm x 1.9 μm Supelco Analytical Column (Sigma-Aldrich), with the flow rate of 0.5 mL min^{-1}. A gradient of 0.1% formic acid in LC-MS-grade water (eluent A) and 0.1% formic acid in acetonitrile (eluent B) was set up as follows: 0% B at 0 min, linear gradient to 100% B at 7 min, and linear gradient to 0% B at 11 min. Mass spectral parameters were set as follows: negative spray voltage 3500 V, capillary temperature 300°C, sheath gas 35 (arbitrary units), aux gas 10 (arbitrary units), and probe heater temperature 200°C

652 with an HESI probe. Full-scan mass spectra were collected (R:35000 full width at half
653 maximum, *m/z* 200; mass range: *m/z* 50 to 750) in negative mode. Excalibur 3.0 software was
654 used to quantify peak areas using a SIM chromatogram measured for *m/z* 240. The relative
655 DIM2BOA content of each sample was estimated from the ratio of the DIM2BOA peak area
656 (mass range of *m/z* 240.0-240.2 and retention time 2.25 min) relative to 2-benzoxazolinone (mass
657 range of *m/z* 134.0-134.2 and retention time 3.26 min), which was used as an internal standard.
658
659 **Chemical Treatment on Plants**
660 OPDA (50 µM) dissolved in 0.1% DMSO was used for exogenous application on maize plants.
661 Control plants were sprayed with 0.1% DMSO. Twenty-four hours after treatment, 10 adult
662 apterous CLAs were introduced and clip-caged on the leaves. Twenty-four hours after CLA
663 feeding, tissues were harvested and processed for RNA isolation. DDG (1 mM; Sigma-Aldrich)
664 dissolved in water was exogenously sprayed on Mp708 and Tx601 plants. Control plants were
665 sprayed with water. Twenty-four hours after spraying, plants were infested with five adult
666 apterous CLAs, and aphid numbers were counted after 4 days. For monitoring *Tdy2* gene
667 expression levels after DDG treatment, plants were sprayed with DDG and water (control).
668 Twenty-four hours after treatment, leaf tissues were harvested for RNA extraction and
669 subsequent RT-qPCR. The Mp708 plants that received coapplication of OPDA and DDG for
670 bioassay and EPG feeding experiments were first sprayed with 50 µM OPDA and then sprayed
671 with 1 mM DDG 3 to 4 h later. Twenty-four hours after DDG spraying, the plants were used for
672 aphid bioassays or EPG experiments.
673
674 **Phytohormone Quantification**
675 Plants were treated with 50 µM OPDA as described above. Control plants were sprayed with
676 0.1% DMSO or did not receive any treatment. Twenty-four hours after treatment, leaf tissues
677 were collected, weighed, and flash-frozen in liquid nitrogen. The tissue samples were ground
678 using a 2010 Geno/Grinder (SPEX SamplePrep) for 40 seconds at 1,400 strokes min^{-1} under
679 liquid nitrogen conditions. The phytohormone analysis was carried out by the Proteomics &
680 Metabolomics Facility at the Center for Biotechnology, University of Nebraska-Lincoln. The
681 ground tissue was dissolved in cold methanol:acetonitrile (50:50, v/v) spiked with deuterium-
682 labeled internal standards (D2-JA; TCI America). After centrifugation at 16,000*g*, the

683 supernatants were collected, and extraction of the pellet was repeated. The supernatants were
684 pooled and dried down using a speed-vac. The pellets were redissolved in 200 µL of 15%
685 methanol. For LC separation, the ZORBAX Eclipse Plus C18 column (2.1 mm × 100 mm;
686 Agilent) was used at a flow rate of 0.45 mL/min. The gradient of the mobile phases A (0.1%
687 acetic acid) and B (0.1% acetic acid/90% acetonitrile) was 5% B for 1 min, to 60% B in 4 min, to
688 100% B in 2 min, hold at 100% B for 3 min, to 5% B in 0.5 min. The Shimadzu LC system was
689 interfaced with a Sciex QTRAP 6500+ mass spectrometer equipped with a TurboIonSpray (TIS)
690 electrospray ion source. Analyst software (version 1.6.3) was used to control sample acquisition
691 and data analysis. The QTRAP 6500+ mass spectrometer was tuned and calibrated according to
692 the manufacturer's recommendations. The hormones were detected using MRM transitions that
693 were optimized using standards. The instrument was set up to acquire data in positive and
694 negative ion switching modes. For quantification, an external standard curve was prepared using
695 a series of standard samples containing different concentrations of unlabeled hormones and fixed
696 concentrations of the deuterium-labeled standards mixture.
697
698 **Statistical Analyses**
699 The statistical analyses were performed using PROC GLIMMIX in SAS 9.4 (SAS Institute). To
700 evaluate the effect of genotype and treatment, and their interaction, two-way analysis of variance
701 (ANOVA) was used. Pairwise comparisons between treatments were carried out by comparing
702 the means with Tukey's honestly significant difference tests ($P < 0.05$). For different EPG
703 parameters, the mean time spent by aphids on various feeding activities was analyzed using the
704 nonparametric Kruskal-Wallis test ($P < 0.05$).
705
706
707 **ACKNOWLEDGEMENTS**
708 We thank Ming Guo, Fan Yang, and Ezhumalai Muthukrishnan for assistance with callose
709 staining and visualization, and Sathish Natarajan for access to the epifluorescence microscope.
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711 Proteomics & Metabolomics Facility at the Center for Biotechnology, University of Nebraska-
712 Lincoln for phytohormone anlaysis.
713

714 **SUPPLEMENTAL DATA**

715 The following materials are available in the online version of this article.

716

717 **Supplemental Figure S1.** Mp708 provides phloem-based resistance to corn leaf aphids.

718 **Supplemental Figure S2.** RT-qPCR analysis of BX pathway genes in Tx601 and Mp708 plants
719 before and after (24 h) CLA infestation.

720 **Supplemental Figure S3.** Expression of *Tdy2* and *ACS6* transcripts after exogenous application
721 of OPDA on maize genotypes.

722 **Supplemental Figure S4.** Pretreatment of Mp708 plants with callose synthesis inhibitor reduces
723 the expression of *Tdy2*.

724 **Supplemental Figure S5.** Blocking callose synthesis attenuates the resistant phenotype of
725 Mp708 plants.

726 **Supplemental Figure S6.** OPDA treatment did not enhance the levels of JA and JA-Ile in
727 Mp708 plants.

728 **Supplemental Table S1.** CLA feeding activities on the maize Mp708 genotype after various
729 chemical treatments.

730 **Supplemental Table S2.** Primers used for RT-qPCR study.

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745 **Table 1**. CLA feeding activities on the maize Tx601 and Mp708 genotypes.

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CLA feeding activity	Tx601	Mp708	*P* Value
Total duration of pathway phase (PP)	3.64 ± 0.36	4.66 ± 0.58	0.367
Total duration of nonprobing phase (NP)	0.91 ± 0.32	0.88 ± 0.2	0.525
Time to first sieve element phase (f-SEP)	3.23 ± 0.41	2.86 ± 0.34	0.564
Total duration of SEP	2.53 ± 0.31	1.77 ± 0.25	0.031*
Total duration of xylem phase (XP)	0.96 ± 0.26	0.69 ± 0.11	0.335

747

748 Values represent mean time (h) ± SE spent by CLA on various activities in each 8 h of recording
749 (n =12). An asterisk represents a significant difference ($P < 0.05$, Kruskal-Wallis test) in the time
750 spent by CLA for the indicated activity on the Tx601 and Mp708 plants.

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770 **FIGURE LEGENDS**

771

772 **Figure 1.** Mp708 provides phloem-based resistance to CLA. A, Mean time spent by CLA for
773 various activities (PP, pathway phase; NP, nonprobing phase; f-SEP, the time to reach first sieve
774 element phase; SEP, the total duration of SEP; XP, xylem phase) on Tx601 and Mp708 maize
775 genotypes. Each value is the mean ± SE of 12 replications. An asterisk represents a significant
776 difference ($P < 0.05$; Kruskal-Wallis test) in the time spent by CLA for the indicated activity on
777 the Tx601 and Mp708 plants. B, Representative EPG waveform patterns over an 8-h period of
778 CLA feeding on Tx601 and Mp708 maize genotypes.

779

780 **Figure 2.** CLA feeding promotes enhanced callose accumulation in the Mp708 genotype. A,
781 Number of callose spots (± SEM) per mm^2 of leaf tissue in CLA-infested and uninfested leaves
782 at different time points ($n = 3$-4). B, RT-qPCR analysis of *Tdy2* transcripts in uninfested (0 h)
783 and CLA-infested leaves (24 h) on Tx601 and Mp708 maize plants ($n = 4$). Different letters
784 above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's
785 test). Error bars represent ± SEM.

786

787 **Figure 3.** BX or BX-derived metabolites are not significantly altered in CLA-infested Mp708
788 plants. A-E, Comparison of BX derivatives in Tx601 and Mp708 maize genotypes after 0, 6, 12,
789 and 24 h of CLA feeding ($n = 4$). FW, Fresh weight; DIMBOA-Glc, 2,4-dihydroxy-7-methoxy-
790 1,4-benzoxazin-3-one glucoside; HDMBOA-Glc, 2-hydroxy-4,7- dimethoxy-1,4-benzoxazin-3-
791 one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; DIM2BOA-Glc, 2,4-
792 dihydroxy-7,8-dimethoxy-1,4- benzoxazin-3-one glucoside; DIM2BOA, 2,4-dihydroxy-7,8-
793 dimethoxy-1,4-benzoxazin-3-one. Different letters above the bars indicate values that are
794 significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

795

796 **Figure 4.** OPDA pretreatment enhances callose accumulation and heightened resistance to CLA
797 in Mp708 plants. A, Number of callose spots (± SEM) per mm^2 of leaf tissue with (+) and
798 without (-) prior treatment of OPDA and CLA infestation (24 h). Plants that were treated with
799 DMSO (solvent-only control) and plants that did not receive any treatment were used as the
800 negative controls ($n = 3$-4). B, Total number of CLA adults and nymphs recovered 4 days after

infestation of Tx601 and Mp708 plants that were pretreated with OPDA for 24 h. Plants that were treated with DMSO (solvent-only control) and plants that did not receive any treatment were used as the controls. Plants were infested with five adult apterous aphids/plant after 24 h of OPDA treatment (n = 12). For A and B, different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

Figure 5. Blocking callose synthesis attenuates the resistant phenotype of Mp708 plants. Total number of CLA adults and nymphs recovered 4 days after infestation of Mp708 plants that were pretreated with either 2-deoxy-D-glucose (DDG), OPDA, or coapplied with OPDA and DDG for 24 h. Plants that were treated with water and DMSO (solvent-only controls) and plants that did not receive any treatment were used as the controls. Plants were infested with five adult apterous aphids/plant after 24 h of chemical/water treatment. Values represent the mean ± SEM of CLA numbers (n = 12). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test).

Figure 6. OPDA application enhances the expression of ethylene biosynthesis and receptor genes and *mir1* transcripts in Mp708 plants. RT-qPCR analysis of *ACS2* (A), *ACS6* (B), *ACO15* (C), *ERS14* (D), and *mir1* (E) in Mp708 leaves before (-) and after (+) OPDA and CLA infestation (24 h). Plants treated with DMSO (solvent-only control) and plants that did not receive any treatment were used as the negative controls (n = 3-4). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

Figure 7. MeJA pretreatment did not significantly alter callose accumulation in Mp708 plants. The number of callose spots (± SEM) per mm^2 of leaf tissue with and without prior treatment of MeJA and CLA infestation (24 h) on Mp708 plants is shown. Plants treated with 0.1% Tween to dissolve MeJA and plants that did not receive any treatment were used as the negative controls (n = 3-4). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

Figure 8. Maize resistance to CLA is independent of the JA pathway. A, Total number of CLA adults and nymphs recovered 4 days after infestation of wild-type (B73) and JA-deficient (*opr7 opr8*) maize plants that were pretreated with OPDA for 24 h. Plants that were treated with DMSO (solvent-only control) and plants that did not receive any treatment were used as the controls. Plants were infested with five adult apterous aphids/plant after 24 h of OPDA treatment (n = 15 [B73] and n = 6-8 [*opr7 opr8*] for each treatment). B, Number of callose spots (± SEM) per mm^2 of leaf tissue with (+) and without (-) prior treatment of OPDA and CLA infestation (24 h). Plants treated with DMSO to dissolve OPDA and plants that did not receive any treatment were used as the negative controls (n = 3). For A and B, different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

Figure 9. OPDA does not have a direct effect on CLA fecundity. Comparison of CLA numbers on artificial diet supplemented with two different concentrations of OPDA. Diet alone and diet supplemented with DMSO, which was used as a solvent for the OPDA, were used as the controls. For feeding trial bioassays, three adult apterous CLAs were introduced into each feeding chamber and allowed to feed on the diet. The total numbers of aphids (adults and nymphs) in each chamber were counted after 4 days (n = 8). This experiment was conducted twice with similar results. No significant differences were observed among any of the treatments ($P > 0.05$; Tukey's test). Error bars represent ± SEM.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1. Mp708 provides phloem-based resistance to corn leaf aphids (CLA). A, Mean time spent by CLA for various activities (PP, pathway phase; NP, nonprobing phase; f-SEP, the time to reach first sieve element phase; SEP, the total duration of SEP; XP, xylem phase) on B73 and Mp708 maize genotypes. Each value is the mean ± SE of 14 replications. An asterisk represents a significant difference ($P < 0.05$; Kruskal-Wallis test) in the time spent by CLA for the indicated activity on the B73 and Mp708 plants. B, Representative EPG waveform patterns over an 8-h period of CLA feeding on B73 and Mp708 maize genotypes.

Supplemental Figure S2. RT-qPCR analysis of BX pathway genes in Tx601 and Mp708 plants before and after (24 h) CLA infestation (n = 3-4). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

Supplemental Figure S3. Expression of *Tdy2* and *ACS6* transcripts after exogenous application of OPDA on maize genotypes. RT-qPCR analysis of *Tdy2* in Mp708 (A) and Tx601 plants (B) before and after OPDA treatment. C, RT-qPCR analysis of ET biosynthetic pathway gene *ACS6* in Tx601 plants before and after OPDA treatment. Plants treated with DMSO (solvent-only control) and plants that did not receive any treatment were used as the controls. For experiments A-C, n = 3. Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

Supplemental Figure S4. Pretreatment of Mp708 plants with callose synthesis inhibitor reduces the expression of *Tdy2*. RT-qPCR analysis of *Tdy2* in Mp708 leaves before and after 2-deoxy-D-glucose (DDG) treatment for 24 h. Plants that were treated with water (solvent-only control) and plants that did not receive any treatment were used as the controls (n = 3). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

Supplemental Figure S5. Blocking callose synthesis attenuates the resistance phenotype of Mp708 plants. The total number of CLA adults and nymphs recovered 4 days after infestation of Tx601 and Mp708 plants that were pretreated with DDG for 24 h is shown. Plants that were treated with water (solvent-only control) and plants that did not receive any treatment were used as the controls. Plants were infested with five adult apterous aphids/plant after 24 h of DDG treatment. Values represent the mean ± SEM of CLA numbers (n = 12). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test).

Supplemental Figure S6. OPDA treatment did not enhance the levels of JA and JA-Ile in Mp708 plants. Constitutive levels of OPDA (A), JA (B), and JA-Ile (C) in Tx601 and Mp708 genotypes and after treatment with 50 µM OPDA for 24 h on Mp708 plants. Plants treated with DMSO (solvent-only control) and plants that did not receive any treatment were used as the controls (n = 3). FW, Fresh weight. Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

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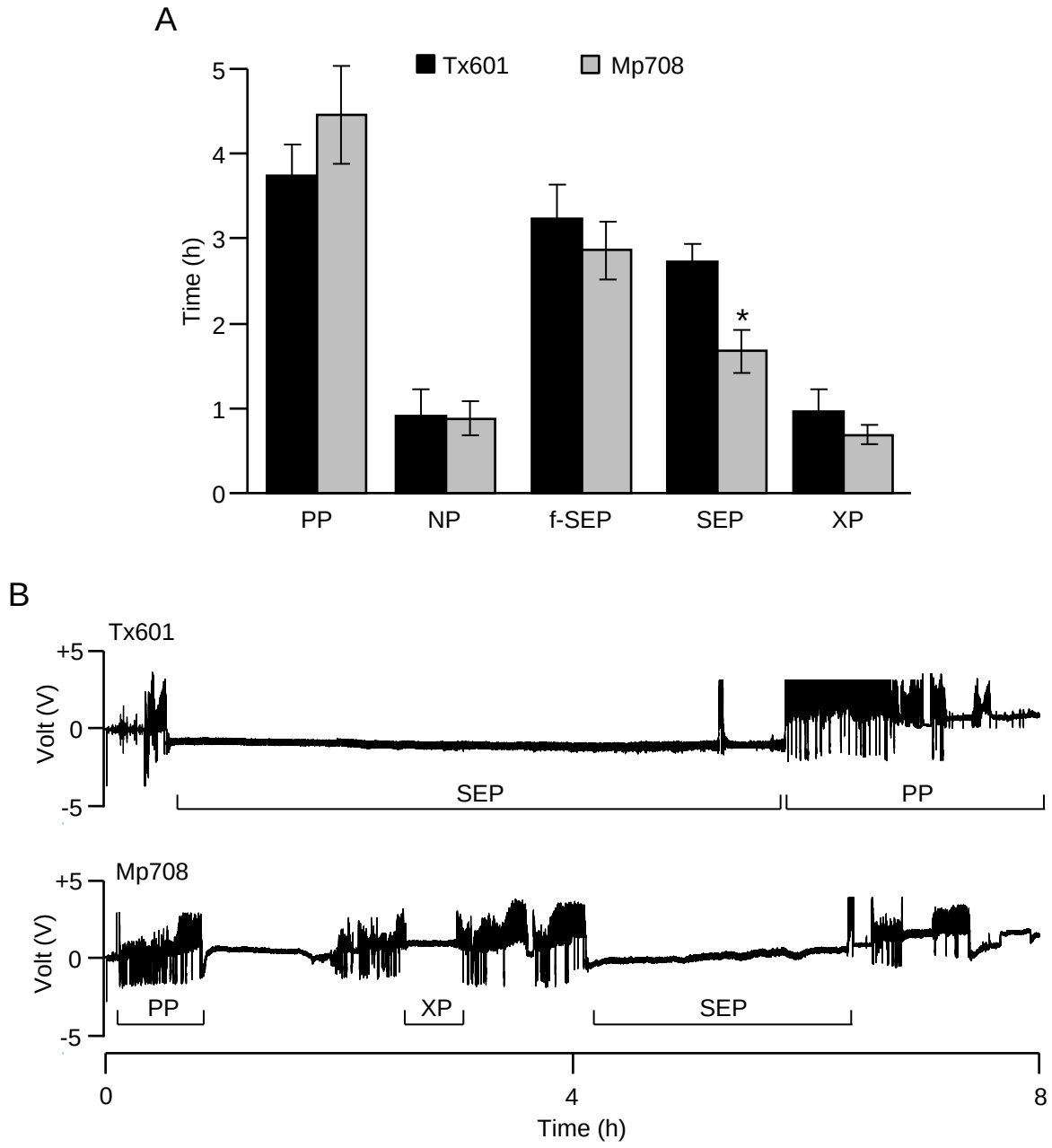


Figure 1. Mp708 provides phloem-based resistance to CLA. A, Mean time spent by CLA for various activities (PP, pathway phase; NP, nonprobing phase; f-SEP, the time to reach first sieve element phase; SEP, the total duration of SEP; XP, xylem phase) on Tx601 and Mp708 maize genotypes. Each value is the mean ± SE of 12 replications. An asterisk represents a significant difference ($P < 0.05$; Kruskal-Wallis test) in the time spent by CLA for the indicated activity on the Tx601 and Mp708 plants. B, Representative EPG waveform patterns over an 8-h period of CLA feeding on Tx601 and Mp708 maize genotypes.

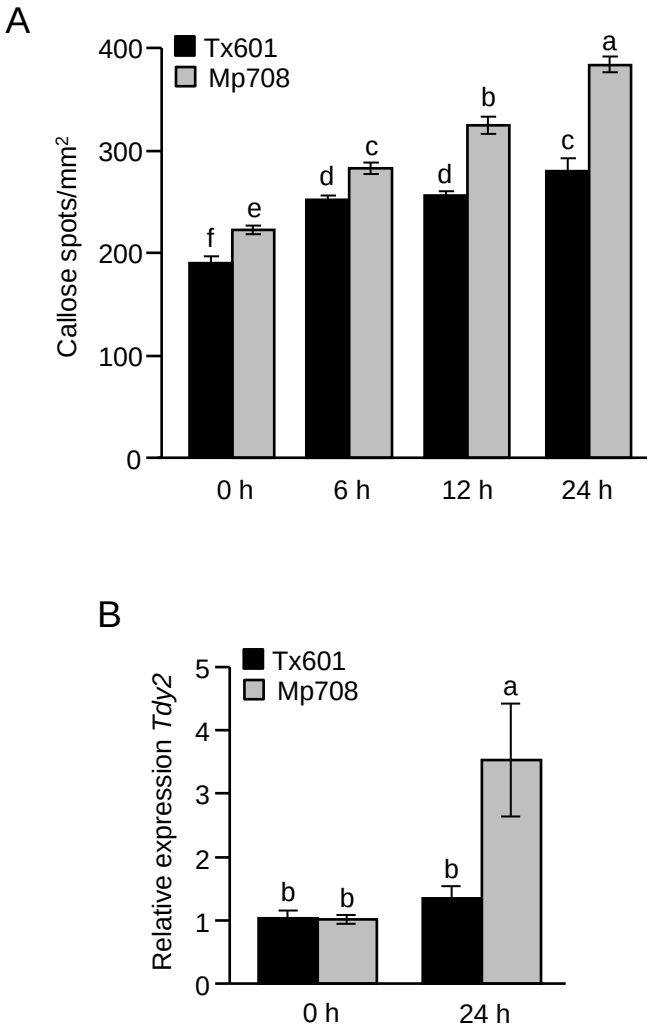


Figure 2. CLA feeding promotes enhanced callose accumulation in the Mp708 genotype. A, Number of callose spots (± SEM) per mm^2 of leaf tissue in CLA-infested and uninfested leaves at different time points (n = 3-4). B, RT-qPCR analysis of *Tdy2* transcripts in uninfested (0 h) and CLA-infested leaves (24 h) on Tx601 and Mp708 maize plants (n = 4). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

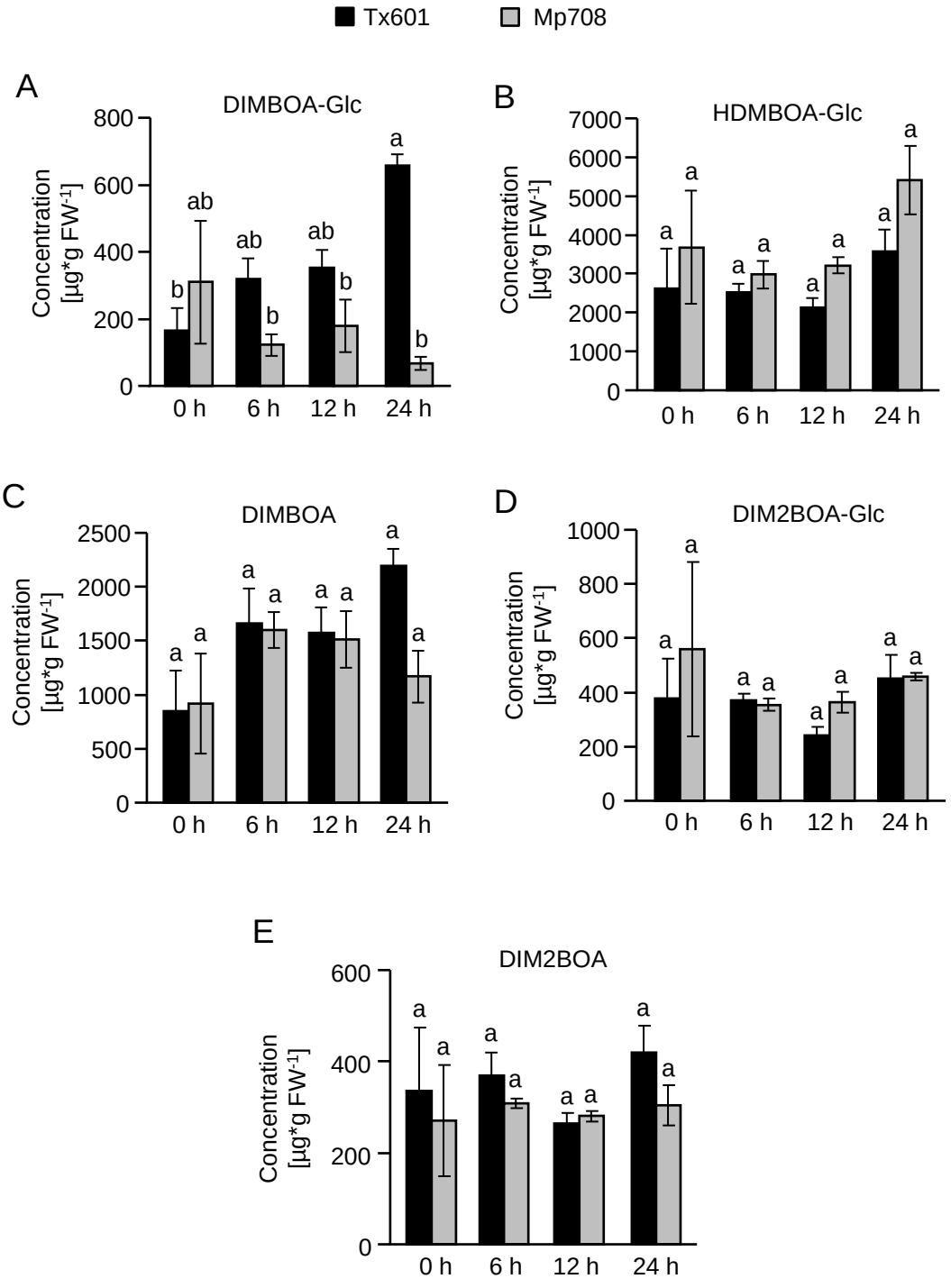


Figure 3. BX or BX-derived metabolites are not significantly altered in CLA-infested Mp708 plants. A-E, Comparison of BX derivatives in Tx601 and Mp708 maize genotypes after 0, 6, 12, and 24 h of CLA feeding ($n = 4$). FW, Fresh weight; DIMBOA-Glc, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside; HDMBOA-Glc, 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; DIM2BOA-Glc, 2,4-dihydroxy-7,8-dimethoxy-1,4- benzoxazin-3-one glucoside; DIM2BOA, 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one. Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

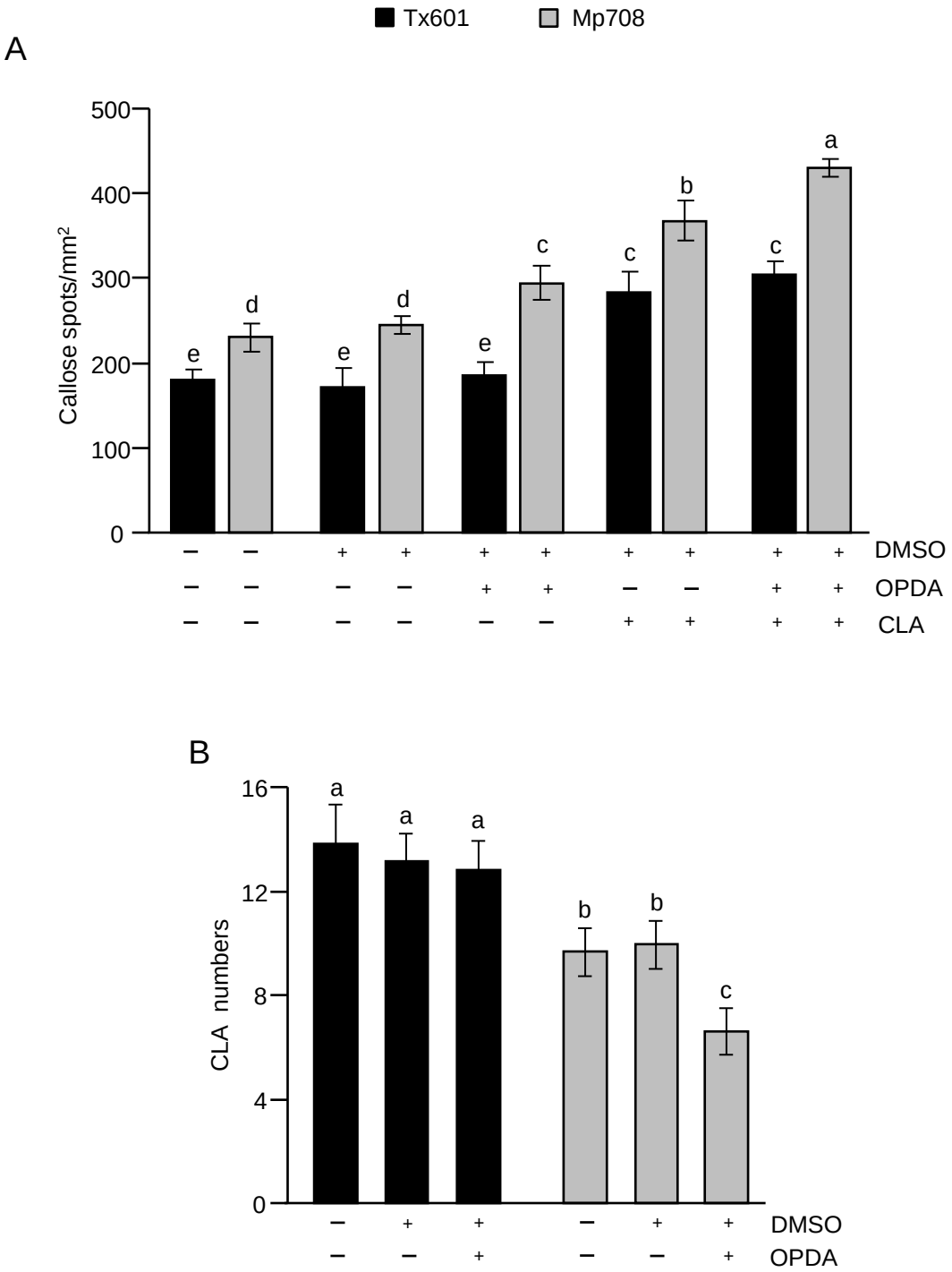


Figure 4. OPDA pretreatment enhances callose accumulation and heightened resistance to CLA in Mp708 plants. A, Number of callose spots (± SEM) per mm^2 of leaf tissue with (+) and without (-) prior treatment of OPDA and CLA infestation (24 h). Plants that were treated with DMSO to dissolve OPDA and plants that did not receive any treatment were used as the negative controls (n = 3-4). B, Total number of CLA adults and nymphs recovered 4 days after infestation of Tx601 and Mp708 plants that were pretreated with OPDA for 24 h. Plants that were treated with DMSO (solvent-only control) and plants that did not receive any treatment were used as the controls. Plants were infested with five adult apterous aphids/plant after 24 h of OPDA treatment (n = 12). For A and B, different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

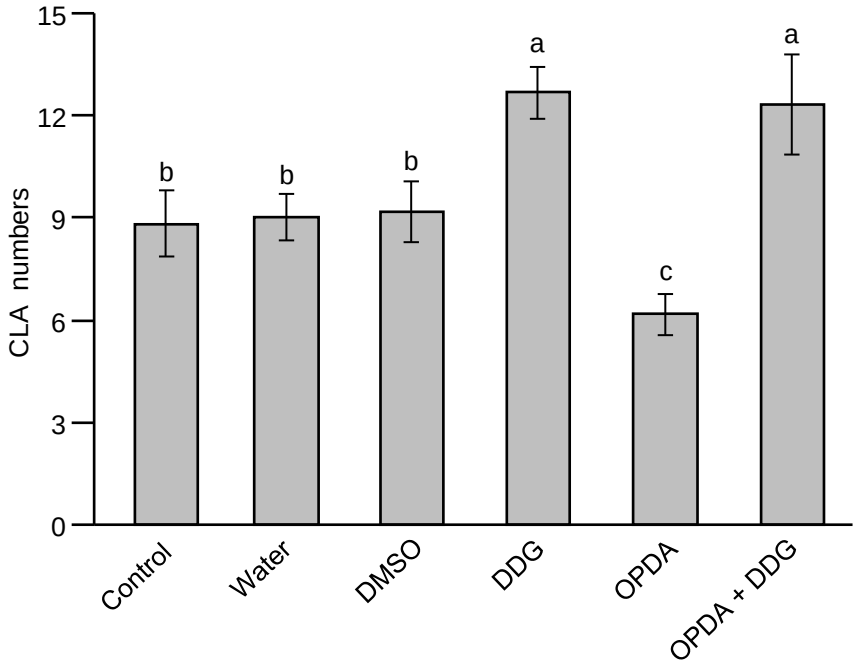


Figure 5. Blocking callose synthesis attenuates the resistant phenotype of Mp708 plants. Total number of CLA adults and nymphs recovered 4 days after infestation of Mp708 plants that were pretreated with either 2-deoxy-D-glucose (DDG), OPDA, or coapplied with OPDA and DDG for 24 h. Plants that were treated with water and DMSO (solvent-only controls) and plants that did not receive any treatment were used as the controls. Plants were infested with five adult apterous aphids/plant after 24 h of chemical/water treatment. Values represent the mean ± SEM of CLA numbers (n = 12). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test).

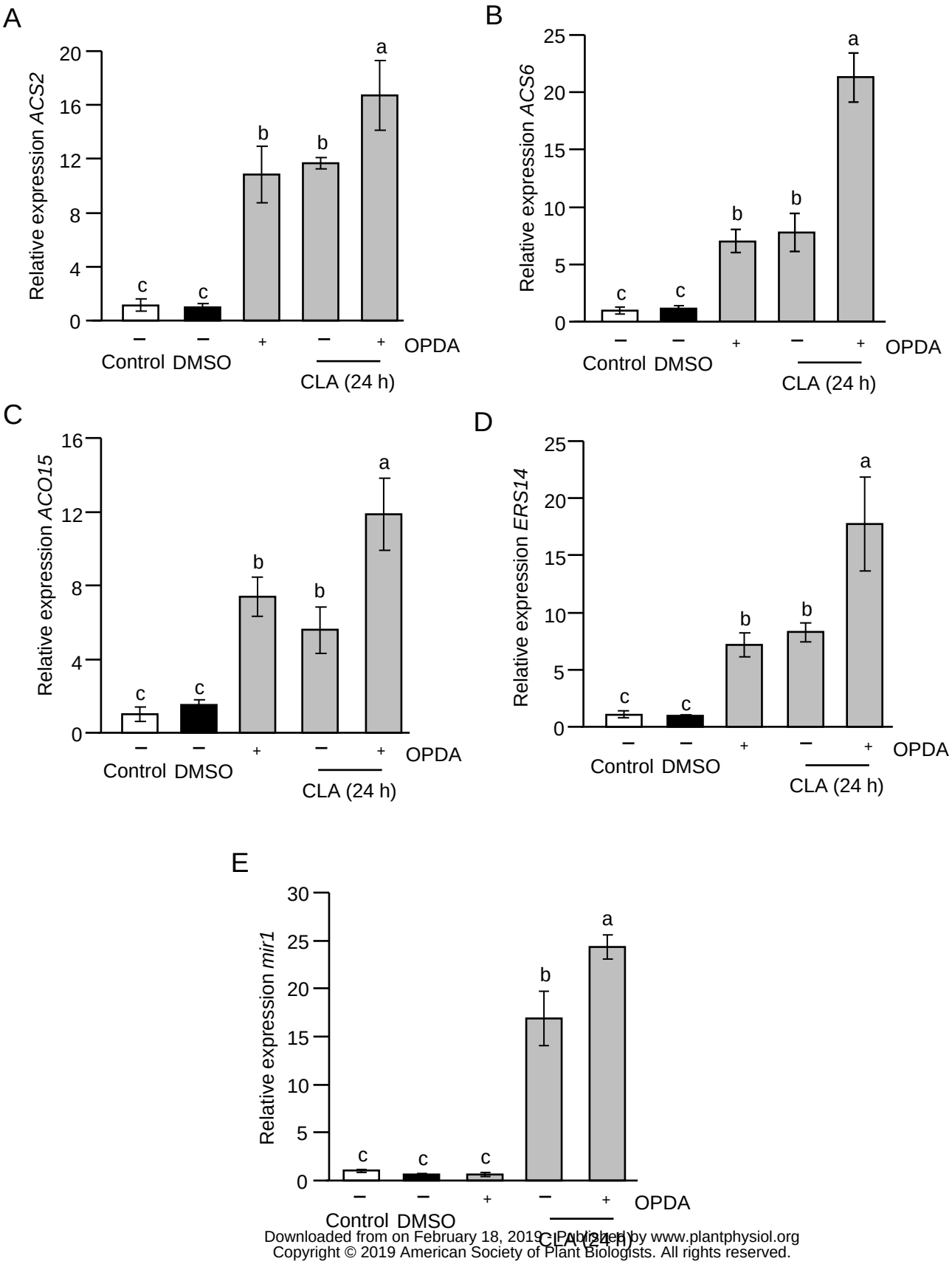


Figure 6. OPDA application enhances the expression of ethylene biosynthesis and receptor genes and *mir1* transcripts in Mp708 plants. RT-qPCR analysis of *ACS2* (A), *ACS6* (B), *ACO15* (C), *ERS14* (D), and *mir1* (E) in Mp708 leaves before (-) and after (+) OPDA and CLA infestation (24 h). Plants treated with DMSO (solvent-only control) and plants that did not receive any treatment were used as the negative controls (n = 3-4). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

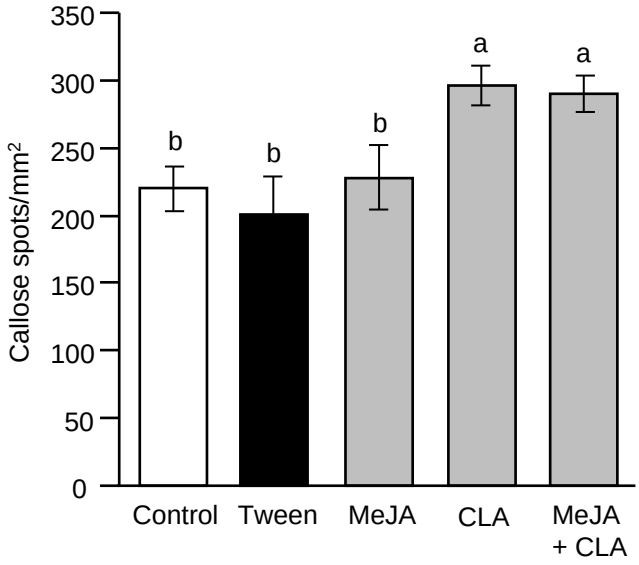
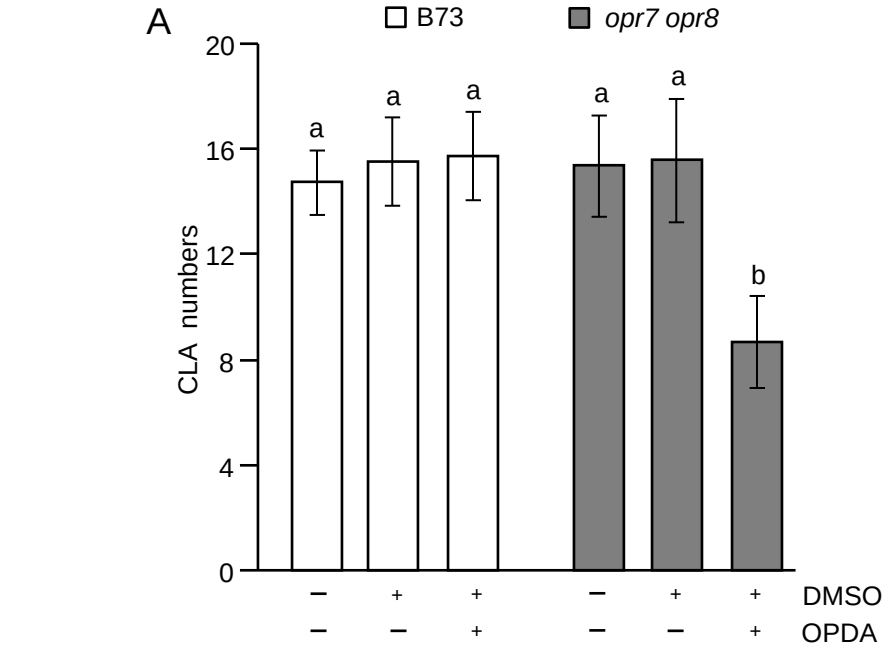


Figure 7. MeJA pretreatment did not significantly alter callose accumulation in Mp708 plants. The number of callose spots (± SEM) per mm^2 of leaf tissue with and without prior treatment of MeJA and CLA infestation (24 h) on Mp708 plants is shown. Plants treated with 0.1% Tween to dissolve MeJA and plants that did not receive any treatment were used as the negative controls (n = 3-4). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.



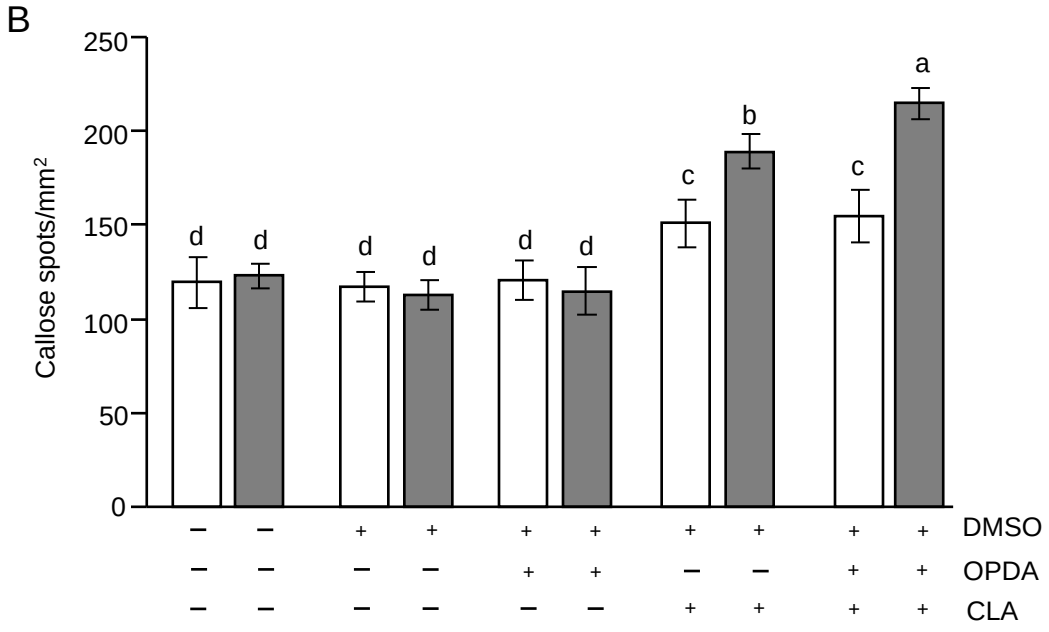


Figure 8. Maize resistance to CLA is independent of the JA pathway. A, Total number of CLA adults and nymphs recovered 4 days after infestation of wild-type (B73) and JA-deficient (*opr7 opr8*) maize plants that were pretreated with OPDA for 24 h. Plants that were treated with DMSO (solvent-only control) and plants that did not receive any treatment were used as the controls. Plants were infested with five adult apterous aphids/plant after 24 h of OPDA treatment (n = 15 [B73] and n = 6-8 [*opr7 opr8*] for each treatment). B, Number of callose spots (± SEM) per mm^2 of leaf tissue with (+) and without (-) prior treatment of OPDA and CLA infestation (24 h). Plants treated with DMSO to dissolve OPDA and plants that did not receive any treatment were used as the negative controls (n = 3). For A and B, different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; Tukey's test). Error bars represent ± SEM.

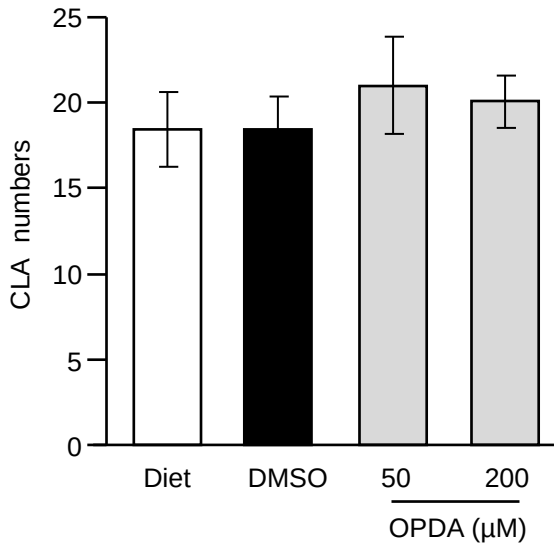


Figure 9. OPDA does not have a direct effect on CLA fecundity. Comparison of CLA numbers on artificial diet supplemented with two different concentrations of OPDA. Diet alone and diet supplemented with DMSO, which was used as a solvent for the OPDA, were used as the controls. For feeding trial bioassays, three adult apterous CLAs were introduced into each feeding chamber and allowed to feed on the diet. The total numbers of aphids (adults and nymphs) in each chamber were counted after 4 days (n = 8). This experiment was conducted twice with similar results. No significant differences were observed among any of the treatments ($P > 0.05$; Tukey's test).). Error bars represent ± SEM.

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