

# Disruption of *OPR7* and *OPR8* Reveals the Versatile Functions of Jasmonic Acid in Maize Development and Defense <sup>W</sup>

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**Here, multiple functions of jasmonic acid (JA) in maize (*Zea mays*) are revealed by comprehensive analyses of JA-deficient mutants of the two oxo-phytodienoate reductase genes, *OPR7* and *OPR8*. Single mutants produce wild-type levels of JA in most tissues, but the double mutant *opr7 opr8* has dramatically reduced JA in all organs tested. *opr7 opr8* displayed strong developmental defects, including formation of a feminized tassel, initiation of female reproductive buds at each node, and extreme elongation of ear shanks; these defects were rescued by exogenous JA. These data provide evidence that JA is required for male sex determination and suppression of female reproductive organ biogenesis. Moreover, *opr7 opr8* exhibited delayed leaf senescence accompanied by reduced ethylene and abscisic acid levels and lack of anthocyanin pigmentation of brace roots. Remarkably, *opr7 opr8* is nonviable in nonsterile soil and under field conditions due to extreme susceptibility to a root-rotting oomycete (*Pythium* spp), demonstrating that these genes are necessary for maize survival in nature. Supporting the importance of JA in insect defense, *opr7 opr8* is susceptible to beet armyworm. Overall, this study provides strong genetic evidence for the global roles of JA in maize development and immunity to pathogens and insects.**

## INTRODUCTION

Jasmonic acid (JA) and its derivatives, such as methyl jasmonate (MeJA) and jasmonoyl-isoleucine (JA-Ile), collectively referred to as jasmonates (JAs), are lipid-derived plant hormones that are common to all higher plant species (Farmer et al., 2003). These compounds play pivotal roles in a number of plant biological processes, such as seed maturation, anther development, root growth, tendril coiling, and responses to biotic and abiotic stresses (Browse, 2009; Avanci et al., 2010). JA biosynthesis is initiated in the chloroplast starting with  $\alpha$ -linolenic acid (C18:3), which is released from membrane lipids by phospholipase A1 (DAD1) and converted to 12-oxo-phytodienoic acid (OPDA) by the consecutive action of lipoxygenase, allene oxide synthase, and allene oxide cyclase (Creelman and Mullet, 1997; Schaller, 2001). OPDA is then transported into the peroxisome, where it is further converted to (+)-7-iso-JA by 12-oxo-phytodienoic acid reductase (OPR) and three  $\beta$ -oxidation steps. (+)-7-Iso-JA often epimerizes into (–)-7-iso-JA or undergoes modifications to produce diverse JA derivatives, including JA-Ile, the bioactive form of JA, which is conjugated to Ile by JA RESISTANT1 (JAR1), a JA-amino acid-conjugating enzyme (Staswick and Tiryaki, 2004). A number of recent studies showed that JA initiates signal transduction upon formation of a SCF<sup>COI1</sup>-

JA-Ile-jasmonate ZIM-domain protein (JAZ) ternary complex (Sheard et al., 2010), in which the JAZ JA repressors are ubiquitinated and subsequently degraded to release the transcription factor MYC2, causing downstream transcription activation of defense responses or developmental regulation (Chini et al., 2007; Thines et al., 2007).

The roles of JA have been extensively studied in *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*). JA biosynthesis mutants, such as *fad3/7/8* (McConn and Browse, 1996), *opr3/dde1* (Sanders et al., 2000; Stintzi and Browse, 2000), *aos/dde2* (Park et al., 2002), and the JA perception mutant *coronatine insensitive1 (coi1)* (Feys et al., 1994) are all male sterile and susceptible to pests and necrotrophic pathogens, with the exception of *opr3*, which is still resistant to the dark-winged fungus gnat *Bradysia impatiens* (Stintzi et al., 2001). The latter finding suggested that the JA precursor OPDA has its own signaling role in defense responses in the absence of JA (Stintzi et al., 2001). A recent study showed that *opr3* is a conditional mutant with residual *OPR3* transcription, which produces a substantial amount of JA upon *Botrytis cinerea* infection (Chehab et al., 2011). Other JA signaling mutants, such as *jar1* (Staswick et al., 1992; Staswick and Tiryaki, 2004) and *jin1/myc2* (Feys et al., 1994; Lorenzo et al., 2004), are fertile but susceptible to pathogens. In tomato, the systemin perception mutant *spr1* (Lee and Howe, 2003), the JA biosynthesis mutant *spr2* (Li et al., 2002), and the JA perception mutant *spr6/jai1* (Li et al., 2004) are defective in wound-induced systemic proteinase inhibitor expression and are susceptible to insect herbivory. Interestingly, in contrast with JA signaling mutants of *Arabidopsis*, the tomato mutants are male fertile, but *jai1* is female sterile (Li et al., 2004), implying that JA has different roles in the reproductive development in different plant species (Li et al., 2004).

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Despite the tremendous economic significance of monocot crops, very little genetic evidence is available for the physiological functions of JA in monocotyledonous species. To date, few monocot JA-deficient mutants have been reported, specifically, the *hebiba* mutant of rice (*Oryza sativa*; Riemann et al., 2003; Riemann et al., 2008) and the *tasselseed1* (*ts1*) mutant of maize (*Zea mays*; Acosta et al., 2009). In *hebiba*, the induction of JA by wounding is impaired and the response of the mesocotyl and the coleoptile to red light was attenuated (Riemann et al., 2003). The *ts1* mutation results in disruption of JA biosynthesis in developing tassel leading to conversion of the tassel inflorescence from staminate to pistillate (Acosta et al., 2009). Also, overexpression of rice *OPR7* in *Arabidopsis* complemented *opr3* defects, indicating rice *OPR7* is a JA-producing enzyme (Tani et al., 2008). Moreover, recombinant *JAR1* and *JAR2* proteins showed JA-Ile-conjugating activity, and *JAR1* and *JAR2* are differentially induced upon wounding or pathogen challenge (Wakuta et al., 2011).

Plant OPRs are classified into two groups (I and II) depending on their substrate specificity (Zhang et al., 2005; Tani et al., 2008). Group II enzymes reduce the JA precursor *cis*-(+)-OPDA (Schaller et al., 2000) and are therefore proposed to be a major enzyme in JA biosynthesis. Alternatively, OPR enzymes in group I have a very low affinity for *cis*-(+)-OPDA and are unlikely to be involved in JA biosynthesis. For example, the *Arabidopsis* genome contains six *OPR* genes (Chehab et al., 2011), of which only *OPR3* encodes an isoform responsible for JA production (Schaller et al., 2000; Stintzi and Browse, 2000), and *Arabidopsis* *OPR1* and *OPR2* have a broad substrate activity and their physiological function remains obscure (Schaller et al., 2000). Although a number of studies have described the genomic composition of *OPRs* (Zhang et al., 2005; Tani et al., 2008) and expression patterns under different environmental stimuli (Engelberth et al., 2007), the biochemical and physiological functions of most plant *OPRs* are still unclear. To dissect *OPR* functions in maize, we generated *Mutator* (*Mu*) insertional mutants in several members of the *OPR* gene family. In this article, we report on the generation and detailed characterization of mutants in *OPR7* and *OPR8*. *opr7 opr8* double mutants have substantially reduced levels of JA accumulation throughout the plant and they display multiple phenotypes, revealing the global function of JA in maize development and defense.

## RESULTS

### Generation of *opr7*, *opr8*, and *opr7 opr8* Mutants

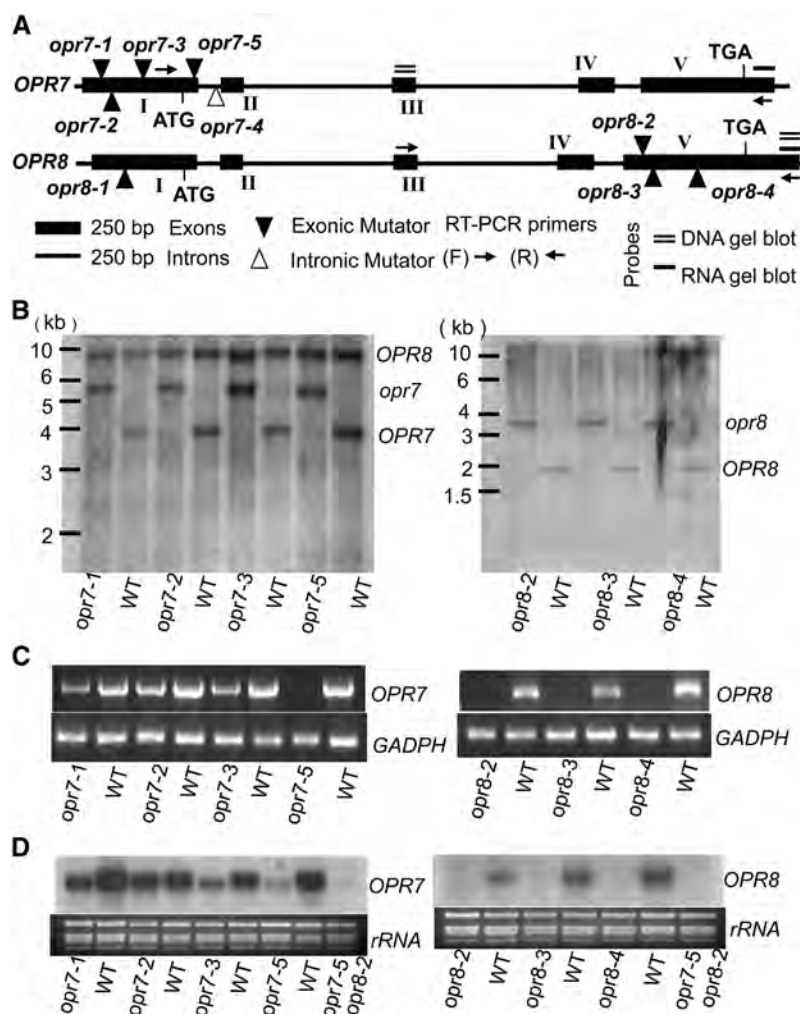
The maize genome harbors eight *OPR* genes (Zhang et al., 2005). Two of them, *OPR7* and *OPR8*, are the closest maize orthologs of *Arabidopsis* *OPR3* (Zhang et al., 2005). *OPR7* and *OPR8* are segmentally duplicated genes located on chromosomes 1 and 4, respectively. They share 94.5% identity in amino acid sequence and 93.8% in their mRNA sequence within the coding sequences. *Mu* transposons, like T-DNA in *Arabidopsis*, are broadly used in maize genetics to generate knockout mutants for genes of interest. Using a reverse genetic resource based on extensive *Mu* transposon population of maize (McCarty and Meeley, 2009), we identified several alleles for both genes. Three independent

mutant alleles of *OPR7* (*opr7-1*, *7-2*, and *7-3*) (Figure 1A; see Supplemental Figure 1 online) and three *OPR8* alleles (*opr8-2*, *8-3*, and *8-4*) (Figure 1A; see Supplemental Figure 2 online) were genetically advanced and functionally characterized in this study. Sequencing of PCR fragments flanking the *Mu* elements revealed that *opr7-1*, *7-2*, and *7-3* alleles had *Mu* insertions in the 5' untranslated region (UTR) of *OPR7* (Figure 1A). The *opr8-2*, *8-3*, and *8-4* alleles are exonic with insertions located in exon V (Figure 1A). The original mutant alleles were backcrossed to the inbred line B73 five to seven times (i.e., to the genetic stage BC5 to BC7, depending on specific alleles). Importantly, while genotyping the *opr7-3* BC3F2 segregating population, a smaller fragment was amplified from a single seedling. Sequencing of this fragment revealed a new allele with a *Mu* element inserted in the first exon of *OPR7*; this newly discovered allele was designated *opr7-5* (Figure 1A; see Supplemental Figure 3 online).

At the BC5 stage, homozygous mutant and wild-type plants for all the alleles were PCR genotyped with gene-specific and *Mu*-specific primers (see Methods). DNA gel blot analysis was used to confirm the presence of *Mu* elements in all of the *OPR7* and *OPR8* alleles (Figure 1B). RT-PCR and RNA gel blot analyses showed that all of the *OPR7* alleles have reduced transcript levels, and *opr8-2*, *8-3*, and *8-4* displayed no detectable transcript (Figures 1C and 1D), indicating that these three alleles are null mutants. The transcripts of *OPR7* in the *opr7-1*, *7-2*, and *7-3* mutants were only slightly less abundant compared with corresponding near-isogenic wild type. The *Mu* insertions of *opr7-1*, *7-2*, and *7-3* are located at 98, 73, and 45 bp before the start codon ATG, respectively. It is likely that the remaining transcripts of *opr7-1*, *7-2*, and *7-3* can be translated into functional *OPR7* enzyme because these transcripts have the entire open reading frame (Figure 1C). Although *OPR7* transcripts are detected by RNA gel blotting in *opr7-5* (Figure 1D), this transcript cannot be translated into a full-length protein because the *Mu* element was located in the first exon and sequencing of this mutant transcript verified that the first exon in *opr7-5* was replaced by a *Mu* element (see Supplemental Figure 3 online). These molecular analyses and, more convincingly, the biochemical and phenotypic analyses presented later suggest that the *opr7-5* allele is an allele with severely reduced gene function. Because *OPR7* and *OPR8* are orthologs of *Arabidopsis* *OPR3*, we hypothesized that they would have redundant functions. For this reason, we created *opr7 opr8* double mutants by crossing all the mutant alleles of *OPR7* with *opr8-2* or by crossing *opr7-5* with all three alleles of *OPR8*. These crosses resulted in the generation of several double mutant combinations: *opr7-1 opr8-2*, *opr7-2 opr8-2*, *opr7-3 opr8-2*, *opr7-5 opr8-2*, *opr7-5 opr8-3*, and *opr7-5 opr8-4*. The described single and double homozygous mutant alleles of *OPR7* and *OPR8* were used for the functional analyses in this study.

### Disruption of *OPR7* and *OPR8* Reduces JA Biosynthesis in Maize

To investigate the function of *OPR7* and *OPR8*, we first studied the expression pattern of these genes. Figure 2A demonstrates that *OPR7* and *OPR8* transcripts accumulated in vegetative and reproductive tissues, including leaf, tassel, ear cop, silk, roots, and mesocotyl, indicating that *OPR7* and *OPR8* may function throughout



**Figure 1.** Analysis of the *Mu* Insertional Alleles of *OPR7* and *OPR8*.

**(A)** Schematic representation of the genomic structure of the maize *OPR7* and *OPR8* genes showing the *Mu* element insertion sites in diverse alleles. *opr7-1*, *7-2*, *7-3*, *7-4*, and *7-5* indicate *Mu*-carrying alleles of *OPR7*. *opr8-2*, *8-3*, and *8-4* are the alleles of *OPR8*. The wild type is the near-isogenic line for individual alleles. The arrows indicate the primer sites for RT-PCR shown in **(C)**, and the hybridizing sites of probes for DNA gel blots and RNA gel blots in **(B)** and **(D)** are shown as “=” and “–”, respectively.

**(B)** DNA gel blot analysis of *OPR7* and *OPR8* alleles. The bands detected by the probes were indicated as *OPR7* or *OPR8*, denoting wild-type (WT) genes, *opr7* indicating a polymorphic band carrying *Mu* insertions in *OPR7*, and *opr8* indicating bands resulting from the insertions in *OPR8*.

**(C)** RT-PCR analysis of *OPR7* and *OPR8* transcripts in the leaves of mutant alleles and near-isogenic wild-type lines using gene-specific primers: 198F and 1512R for *OPR7* and RT-F2 and 1479R for *OPR8*. The maize housekeeping gene *GADPH* was used as a reference gene.

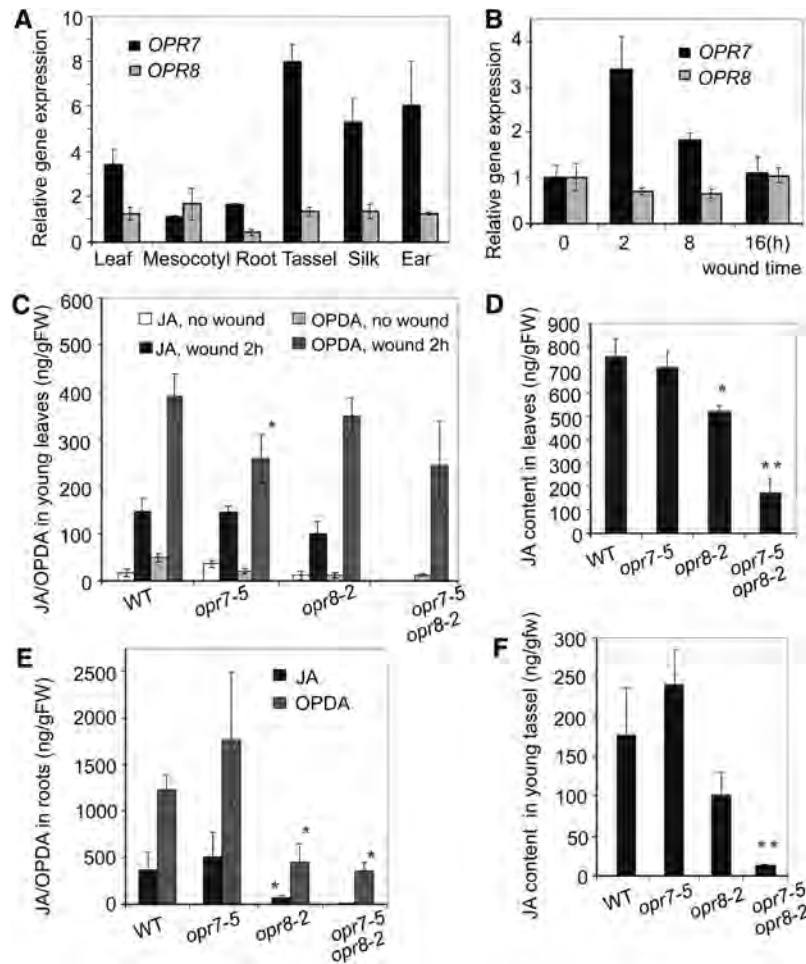
**(D)** RNA gel blot analysis of *OPR7* and *OPR8* transcripts in the leaves of mutant alleles and wild-type lines at the V2 stage.

the plant. Interestingly, mechanical wounding of leaves induced expression of *OPR7* but not *OPR8* (Figure 2B).

To test for the individual and/or collective functions of *OPR7* and *OPR8* in wound-induced JA biosynthesis, we measured JA and OPDA in wild-type, single mutant, and double mutant seedlings 2 h after mechanical wounding, the time at which *OPR7* is induced to maximal level (Figure 2B). The basal levels of JA and OPDA were similar between single mutants and the wild type, whereas JA was not detected in the double mutant. Wound-induced JA and OPDA levels in *opr7-5* or *opr8-2* single mutants were comparable to wild-

type levels (Figure 2C). There were no detectable levels of JA in the double mutant leaves in response to wounding, providing evidence that *OPR7* and *OPR8* are functionally redundant for both basal and wound-induced JA at this stage of plant development. Despite the lack of the *OPR* enzymes that use OPDA as a substrate for JA biosynthesis, OPDA levels in the double mutant remained the same as in the wild type.

To understand the function of *OPR7* and *OPR8* in JA biosynthesis in different organs of adult maize plants, we measured JA content in leaves and tassels at the V12 stage and in roots at



**Figure 2.** Double Mutation in *OPR7* and *OPR8* Greatly Reduced or Abolished JA Biosynthesis in Leaves, Tassel, and Roots.

(A) Transcripts of *OPR7* and *OPR8* in the indicated tissues, as detected by real-time PCR.

(B) Transcripts of *OPR7* and *OPR8* in response to wounding, as detected by real-time PCR.

(C) JA and OPDA contents were measured by LC-MS in wounded (2 h) and nonwounded leaves of *opr7-5*, *opr8-2*, *opr7-5 opr8-2*, and near-isogenic line wild type at the V2 stage. FW, fresh weight.

(D) JA content was measured in the adult plant leaves of *opr7-5*, *opr8-2*, *opr7-5 opr8-2*, and near-isogenic wild-type line. The samples are the top portions of 13th leaf at the V12 stage.

(E) JA and OPDA contents were measured in the roots of the V4 stage plants.

(F) JA content was detected in the young tassel of adult plants at the V12 stage.

Error bars represent sd. Significance at \* $P \leq 0.05$ \*\* $P \leq 0.01$  (see Methods).

the V4 stage. In the 13th leaf of these adult plants, JA level in *opr7-5* mutants was comparable to the wild type, but *opr8* mutants and *opr7-5 opr8-2* double mutants accumulated only 70 and 20% of the wild-type levels, respectively (Figure 2D). These data indicate that, while both *OPR* genes are required for normal JA production, *OPR8* appears to contribute more substantially to JA biosynthesis in the adult maize leaves. Measuring JA content in wild-type leaves, we observed significant age-dependent differences in JA levels (Figures 2C and 2D). This difference between young and adult plants has been consistently observed in many other experiments, suggesting that JA content in maize leaves is developmentally regulated and increases steadily with age. In roots, *opr7-5* had wild-type JA and OPDA levels, but *opr8-2* had

significantly reduced JA and OPDA content (Figure 2E), implying a unique contribution of *OPR8* to JA synthesis in the roots. JA was not detectable in the *opr7-5 opr8-2* roots, and OPDA levels were reduced approximately twofold compared with the wild type. In young tassels (4 to 6 cm long), *opr7-5* and *opr8-2* single mutants had wild-type levels of JA, but the double mutant levels were extremely low (Figure 2F). Collectively, our results show that for the most tissues analyzed, *OPR7* and *OPR8* are functionally redundant, and disruption of both genes causes global reduction of JA in diverse maize organs, while OPDA levels remain essentially unchanged. Unlike other organs tested, *OPR8* appears to have unique function in JA production in the roots and older leaves.

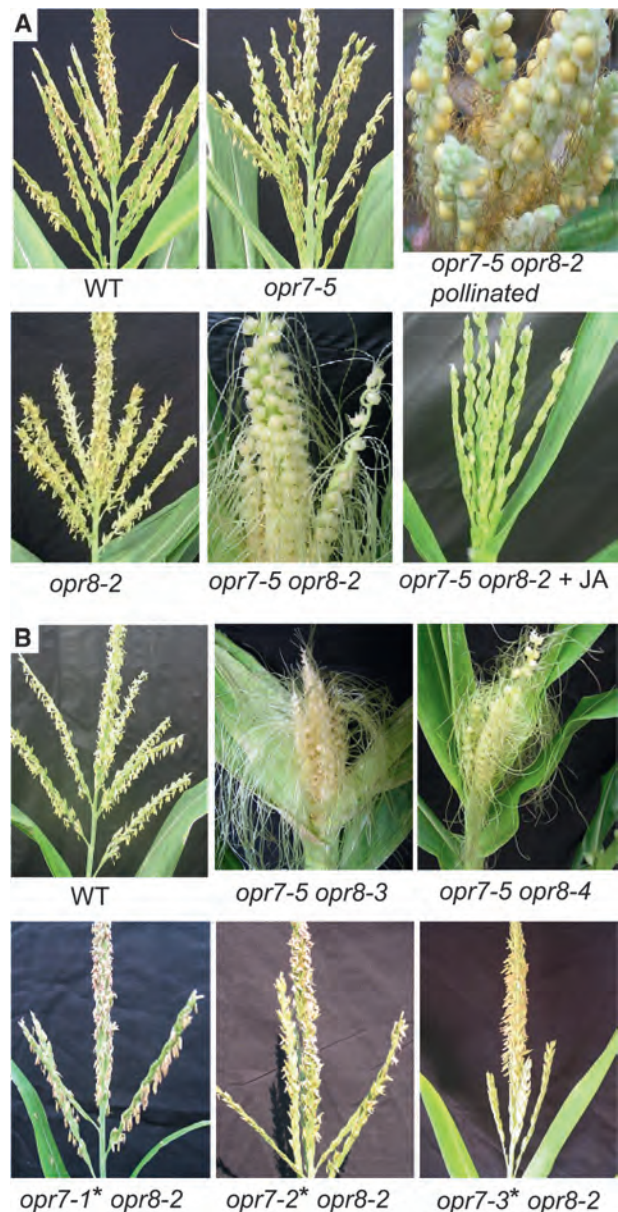
### *opr7 opr8* Displays Phenotypes of Tasselseed, Outgrowth of Numerous Ear Buds, and Elongated Ear Shoots

Field and greenhouse observation of the single mutants did not reveal any noticeable morphological abnormalities, including tassel or ear development. However, populations segregating for the *opr7-5 opr8-2* double mutant showed an interesting flower phenotype in which tassels were feminized and able to bear seeds if pollinated (Figure 3A). Additional plantings of populations segregating for *opr7-5* and *opr8-2* single mutants and *opr7-5 opr8-2* double mutants showed that the tasselseed phenotype was only present in the double mutant lines (Figure 3A), suggesting that both *OPR7* and *OPR8* are required for normal tassel development.

To further confirm that the tasselseed phenotype is due to disruption of both *OPR* genes, we crossed *opr8-2* with the *OPR7* weak alleles *opr7-1*, *7-2*, and *7-3* and the allele with severely reduced gene function, *opr7-5*, with the *OPR8* alleles *opr8-2*, *8-3*, and *8-4*. Double mutant combinations generated from the crosses between *opr7-5* and any of the *OPR8* mutant alleles showed the tasselseed phenotype, whereas the combinations of the leaky *OPR7* alleles with *opr8-2* showed normal tassel morphology (Figures 3A and 3B). These results demonstrated that *opr7-5* is most likely a null allele, and only disruption of both genes leads to tasselseed, indicating that maize plants are functionally dependent on both *OPR7* and *OPR8* for normal tassel development.

Because single mutants with normal JA levels had wild-type tassel morphology, the tasselseed phenotype appeared to be associated with dramatically reduced JA levels in the developing tassel tissues of *opr7-5 opr8-2* (Figure 2F), strongly implicating a role for JA in tassel formation. Indeed, Acosta et al. (2009) reported that the *ts1* mutant was defective in a lipoxygenase enzyme responsible for JA biosynthesis in the tassel (Acosta et al., 2009). To confirm that the lack of JA in the developing *opr7 opr8* tassel is the cause for tasselseed formation, we performed a JA complementation experiment on *opr7-5 opr8-2* according to Acosta et al. (2009). The feminized tassel of *opr7-5 opr8-2* was reversed into the normal tassel morphology by JA treatment (Figure 3A). Our results provide yet another line of genetic evidence to demonstrate that JA is required for normal tassel development.

Having shown a strong role for JA in male sex determination, we then asked if JA affects the formation of the female inflorescence in maize: the ear. Modern domesticated maize has five to seven reproductive axillary buds located in the axil of a leaf positioned at a stem node, only a few of which become fertile ears to accept pollen (Baba and Yamazaki, 1996). In our study, we observed that *opr7 opr8* mutants (Figures 4B to 4D) make three to four abnormally elongated ears compared with the two produced by wild-type plants (Figure 4A), indicating that double mutants had more reproductive buds that develop into ear shoots. This was further confirmed by stripping off all the leaves on the V12 stage plants, which revealed that all the axillary buds on the stem nodes of the double mutants (*opr7-5 opr8-2*, *opr7-5 opr8-3*, and *opr7-5 opr8-4*) showed reduced growth repression in comparison to the wild type (Figures 4F to 4H). Remarkably, all double mutants formed buds even on the

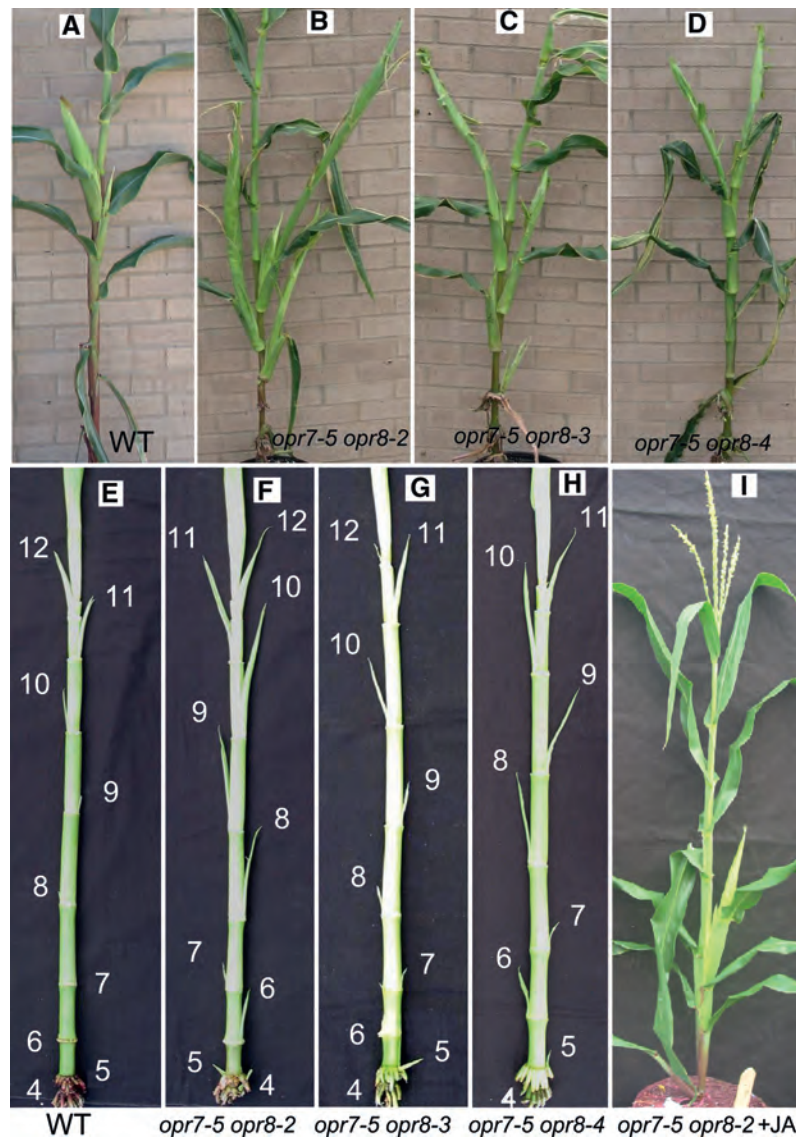


**Figure 3.** JA Deficiency Results in the Tassel Feminization Phenotype Tasselseed in Maize.

(A) Tassel morphologies of wild-type (WT), *opr7-5* and *opr8-2* single mutant, and *opr7-5 opr8-2* double mutant plants. The feminized tassel of *opr7-5 opr8-2* can produce seeds if pollinated and can be complemented with JA to restore a normal tassel phenotype.

(B) In addition to *opr7-5 opr8-2*, other double mutant combinations, *opr7-5 opr8-3* and *opr7-5 opr8-4*, also showed the tasselseed phenotype. Combinations of *opr7-1*, *opr7-2*, or *opr7-3* (\*transcription leaky alleles of *OPR7*) with *opr8-2* displayed wild-type tassel morphology.

brace root nodes. By contrast, wild-type plants have two to three derepressed axillary buds (Figure 4E), but the growth of the remaining axillary buds, visible under closer inspection (see Supplemental Figure 4 online), remains suppressed. This observation clearly demonstrated that *opr7 opr8* mutants have lost



**Figure 4.** JA Deficiency Results in Abnormally Elongated Ear Shoots Phenotype in Maize.

The wild-type (WT; **A**) plant showed normal ear shoots and normal outgrowth of ear buds (**E**). *opr7 opr8* double mutant combinations, *opr7-5 opr8-2* (**B**), *opr7-5 opr8-3* (**C**), and *opr7-5 opr8-4* (**D**), displayed multiple elongated ear shoots. Exogenous JA application (see Method) to *opr7-5 opr8-2* (**I**) rescued the phenotype of elongated ear shoots as well as the tasselseed phenotypes. Double mutant combinations, *opr7-5 opr8-2* (**F**), *opr7-5 opr8-3* (**G**), and *opr7-5 opr8-4* (**H**), showed that all the axillary buds (4th to 12th) were elongated at the V12 stage, whereas the wild type had only three normally elongated (12th, 11th, and 10th) buds, two of which will usually develop into ears.

suppression of axillary bud outgrowth. Interestingly, this multiple ears phenotype was not observed on *ts1* mutants, which appear to have reduced JA production specifically in the tassel (Acosta et al., 2009). These observations suggest that *opr7 opr8* produces more ears due to systemic JA deficiency.

In addition to producing multiple ears, as seen in Figures 4B to 4D, the shank of the double mutants has lost elongation control. The first ear shoot of the mutants ranges from 60 to 80 cm long (Figures 4B to 4D), but the first ear shoot of the wild type averages only ~20 cm (Figure 4A). Exogenous application of 1 mM JA to the apical whorl rescued both tasselseed and the

multiple ears with elongated shank phenotypes (Figure 4I). Our data strongly suggest that JA controls ear outgrowth and elongation of female reproductive organs rather than determining the sex of female inflorescence, indicating that JA is also necessary for normal ear development in maize.

#### JA Promotes Natural Leaf Senescence in Maize

Currently, the role for JA in leaf senescence remains unclear, as a number of studies have reported conflicting results (Schenk et al., 2000; He et al., 2002; Castillo and León, 2008; Seltmann

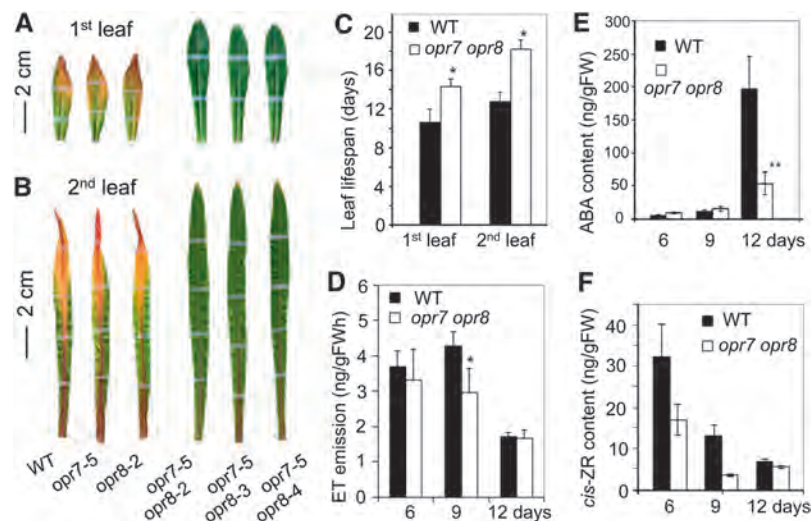
et al., 2010; Shan et al., 2011). In this study, we noticed that the first and second leaves (the oldest leaves of the 2- to 3-week-old seedlings) of *opr7 opr8* senesced substantially later than the first and second leaves on wild-type plants (Figures 5A and 5B). This phenotype was consistently observed for all three double mutant combinations (i.e., *opr7-5 opr8-2*, *opr7-5 opr8-3*, and *opr7-5 opr8-4*) but not for any single mutants (Figures 5A and 5B). The lifespan of the first and second leaves of *opr7-5 opr8-2* was 4 and 5 d longer, respectively, than the wild type in our laboratory conditions (Figure 5C). Collectively, these data indicate that JA is necessary for normal leaf senescence progression as eventually senescence does occur in the *opr7 opr8* mutants.

Besides JA, other plant hormones, such as cytokinins (CKs), ethylene, and abscisic acid (ABA), are well-known regulators of leaf senescence (Lim et al., 2007). To find out if JA deficiency affects production of these other hormones during leaf senescence, we measured the contents of ethylene, ABA, and CKs in wild-type and double mutant leaves during progression of senescence. Ethylene emission was only slightly lower in *opr7 opr8* than the wild type (Figure 5D). However, ABA production was dramatically lower in senescing *opr7 opr8* leaves compared with the wild type (Figure 5E), indicating the key role for JA in the regulation of ABA biosynthesis during senescence. Opposite to our original prediction that *opr7 opr8* may have higher endogenous CK content since CK accumulation was frequently reported to cause delayed leaf senescence (Lim et al., 2007), we found that CK levels in the wild type were higher than in the double mutant in senescing leaves. Out of

the 23 known forms of CK, only 10 were detected (see Supplemental Figure 5 online). Interestingly, the putative bioactive form, *cis*-ZR, is consistently lower in the first leaves of *opr7 opr8* compared with the wild type (Figure 5F). This effect was seen in the first and second leaves for all forms of CKs detected, whether for the bioactive form, the precursors, or the glucosyl conjugate (see Supplemental Figure 5 online), indicating that the entire CK pathway was downregulated in *opr7 opr8* due to JA deficiency. With these data we conclude that the CK pathway depends on the JA signal in maize, but the antisenesescence effect of CKs is not required to antagonize and delay leaf senescence.

### JA Is a Positive Regulator of Anthocyanin Pigmentation in Maize Organs

Anthocyanin pigmentation in maize, especially in kernels, has been extensively studied both genetically and biochemically (Holton and Cornish, 1995), but the involvement of JA in the regulation of anthocyanin biosynthesis in monocots remains unclear. To examine anthocyanins, we carefully monitored mutant plants for any alteration in the pigmentation pattern of diverse organs, such as leaf blade, stem node, brace roots, and kernels. No difference was observed between the wild type and the *opr7* or *opr8* single mutant, and the brace root pigmentation of *opr7-5 opr8-2* double mutants was completely lost (Figure 6A). This phenotype was complemented by exogenous application of 1 mM JA to the leaf whorl (Figure 6A). Other double mutant



**Figure 5.** *opr7 opr8* Double Mutants Display Delayed Leaf Senescence.

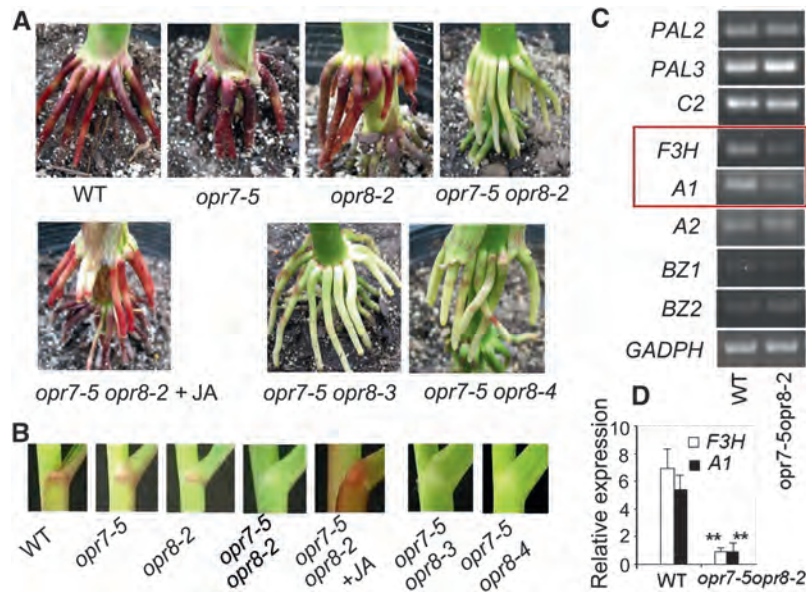
(A) and (B) The first (A) and second (B) leaves of *opr7 opr8* combinations showed a delayed leaf senescence phenotype in comparison with the wild type (WT) and single mutants. Pictures of the first and second leaves were taken at 10 and 11 d after planting, respectively.

(C) The lifespan of the first and second leaves of *opr7-5 opr8-2* and the wild type. Leaf lifespan was defined as number of days from the date when the leaf is fully expanded to the date when 50% of the leaf area had senesced.

(D) Ethylene emission in the first leaf of *opr7-5 opr8-2* and the wild type at 6 and 9 d after full expansion. FW, fresh weight.

(E) ABA content was analyzed in the first leaf of *opr7-5 opr8-2* and wild-type plants at 6, 9, and 12 d after full expansion.

(F) *cis*-Zeatin 9-riboside (*cis*-ZR) content was measured in the first leaf of *opr7-5 opr8-2* and wild-type plants at 6, 9, and 12 d after full expansion. See other forms of CKs in Supplemental Figure 5 online. Error bars represent SD.



**Figure 6.** The *opr7 opr8* Double Mutant Shows Lack of Anthocyanin Pigmentation in Brace Roots and Leaf Collar.

**(A)** Anthocyanin pigmentation in brace roots of the genotypes used in this study. For the JA complementation assay, 1 mL of 1 mM JA solution was added to the whorl of *opr7-5 opr8-2* on eight different occasions (once every 2 d; see Methods). WT, the wild type.

**(B)** Anthocyanin pigmentation in the auricles of genotypes used in this study.

**(C)** and **(D)** Expression analysis of anthocyanin biosynthesis genes by RT-PCR **(C)** and quantitative analysis of *F3H* and *A1* by real-time PCR in the brace roots of double mutant and wild-type plants **(D)**. The primers and gene IDs are listed in Supplemental Table 1 online. Error bars represent sd. Significance at \* $P \leq 0.05$ /\*\* $P \leq 0.01$ .

combinations (*opr7-5 opr8-3* and *opr7-5 opr8-4*) displayed the same phenotype as *opr7-5 opr8-2* (Figure 6A), providing strong evidence that JA is required for the regulation of anthocyanin biosynthesis in brace roots of maize.

To better understand how JA affects anthocyanin biosynthesis (Holton and Cornish, 1995) in maize brace roots, we tested the expression of several anthocyanin biosynthesis genes using RT-PCR. There was no difference in transcript levels for expression of *PAL2*, *C2*, *A2*, *BZ1*, and *BZ2* (see Supplemental Table 1 online for a list of all the genes tested) between the mutant and the wild type (Figure 6C), but *PAL3* displayed higher expression in mutant than the wild type. The two genes that appear reduced in their expression levels encode anthocyanin biosynthesis enzymes flavanone 3-hydroxylase (*F3H*) and dihydroflavonol reductase (*A1*) (Figure 6C). Real-time PCR quantification of these two genes showed that the expression levels of *F3H* and *A1* were 7 and 5 times higher in the wild type compared with *opr7-5 opr8-2*, respectively (Figure 6D). With these results, we conclude that the *F3H* and *A1* genes are partially JA dependent for normal anthocyanin production in brace roots.

Besides brace roots, JA appears to regulate the synthesis of anthocyanin pigment in other maize tissues. In young seedlings, the auricles of the first and second leaves of all *opr7 opr8* knockout combinations lost pigmentation, but the auricles of single mutant and wild-type plants were purple (Figure 6B). The auricle color of *opr7-5 opr8-2* can revert to wild-type levels in 48 h by spraying *opr7-5 opr8-2* leaves with 50  $\mu$ M JA (Figure 6B), further demonstrating that auricle color of maize seedlings depends on JA.

### *opr7 opr8* mutants Are Highly Susceptible to the Oomycete *Pythium* sp

While growing segregating populations to identify the double mutants under field conditions, we consistently observed that none of the surviving mature plants were *ts*, suggesting that the double mutants did not survive in the field. Therefore, we hypothesized that *opr7 opr8* double mutants may be extremely susceptible to soil-borne pathogens. To test this hypothesis, we planted *OPR7opr7-5/opr8-2 opr8-2* F2 seeds, which segregated 1:3 for double mutants (*opr7-5 opr7-5/opr8-2 opr8-2*) and other genotypes (*OPR7 OPR7/opr8-2 opr8-2* or *OPR7 opr7-5/opr8-2 opr8-2*) in the Texas AgriLIFE Experiment Station field. At the V1 to V3 stage, some of the plants wilted and died, and some of the seedlings did not emerge from the soil. These plants were double mutants, as determined by genotyping with PCR using *OPR7*- and *OPR8*-specific primers. The incidence of pre- and post-emergence damping-off was 27% of the planted seeds ( $n = 103$ ) (see Supplemental Figure 6A online). The proportion of plants succumbing to damping-off coincides with the proportion of double mutants occurring in the F2 segregating populations ( $\chi^2 = 0.1585$ ,  $P < 0.05$ ). These data indicate that *opr7 opr8* is not viable under normal field conditions.

To test the viability of *opr7 opr8* in nature at postgermination stages, *opr7-5 opr8-2* and wild-type plants were grown in sterile soil for 14 d and then transplanted in the field. All of the *opr7-5 opr8-2* plants wilted 6 d after the transfer, whereas wild-type plants grew well (Figure 7A). Eleven days after transplanting, all the *opr7-5 opr8-2* plants had died, while wild-type plants continued to display

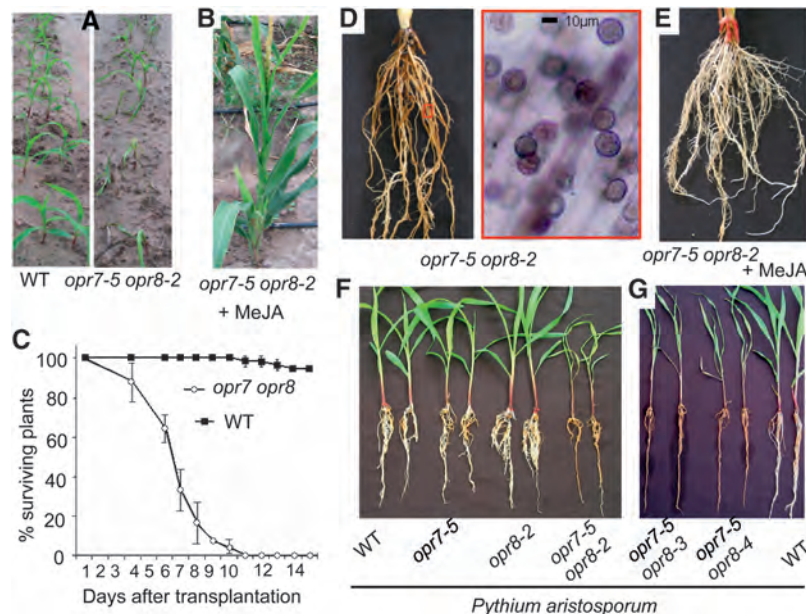


normal growth (Figure 7C). Applying 100 mL of 500  $\mu$ M MeJA to the rhizosphere of *opr7-5 opr8-2* plants in the field every 2 d prevented them from dying (Figure 7B). These field experiments suggest that *opr7 opr8* was compromised in its defense against soil-borne pathogens because of its inability to produce JA.

Because *opr7 opr8* plants cannot grow in the field, we attempted to grow them in sterile and nonsterile artificial soil mixes. In sterile soil, all the double mutants survived. However, in nonsterile mixes, all *opr7 opr8* individuals died before flowering (see Supplemental Figure 7 online). Double mutant plants remained healthy following a rhizosphere drench with 500  $\mu$ M MeJA, again indicating that defense against this soil-borne disease depends on JA production in the plants (see Supplemental Figure 7 online). After checking the roots of wilted and JA-treated *opr7-5 opr8-2* plants, we found that the former were necrotic and the latter were healthy (Figures 7D and 7E). Numerous oospores were observed in the necrotic roots (Figure 7D) and, following isolation and purification, were later identified as *Pythium aristosporum* by cloning and sequencing the internal transcribed spacer (ITS) region of nuclear rRNA genes using primers ITS1 and ITS4 (Paul et al., 2006) and also by characteristics of the oospores and antheridia. The isolates from the necrotic roots of diseased *opr7 opr8* plants in the field were also identified as *P. aristosporum*. Following inoculation of this species to the roots of single mutants, double mutants, and wild types, the *opr7-5 opr8-2* plants were blighted and the roots rotted after 5 d, but single mutant and wild-type plants were

asymptomatic through the completion of the experiment (Figure 7F). Other double mutant combinations were as susceptible as *opr7-5 opr8-2* to *P. aristosporum* (Figure 7G). In summary, our results provide strong evidence that JA biosynthesis in maize is essential for maize immunity against *Pythium* damping-off disease.

In contrast with defenses against foliar pathogens, little is known about molecular events in response to root-rotting pathogens, such as *Pythium* spp. To identify candidate JA-regulated genes that may be required for root innate immunity to *Pythium* disease, expression of 23 selected JA synthesis/signaling (such as *LIP-OXYGENASE* [*LOX*], *ALLENE OXIDE SYNTHASE* [*AOS*], *ALLENE OXIDE CYCLASE* [*AOC*], *JAZ*) or JA-regulated defense genes were tested in roots in response to *P. aristosporum* infection using RT-PCR. In wild-type plants, nine of the selected genes, including *LOX3*, *LOX4*, *PR1*, and *PR5*, were highly induced following *Pythium* infection. Furthermore, genes involved in JA biosynthesis, *LOX8* and *AOC*, or in JA response, *JAZ5* and *LOX2*, and the maize proteinase inhibitor *MPI* were constitutively expressed during infection with *P. aristosporum*. However, in *opr7-5 opr8-2* double mutants, these inducible and constitutively expressed genes, with the exception of *PR1* and *PR4*, were dramatically impaired in their expression in response to the infection (see Supplemental Figure 8 online). Moreover, *LOX2*, *LOX5*, *JAZ4*, and *JAZ8* appear to be completely dependent on JA for expression in roots. Taken together, our data strongly suggest that JA mediates the defensive



**Figure 7.** The *opr7 opr8* Double Mutant Is Nonviable in the Field Soil, and Nonsterile Artificial Mixes Due to Damping-Off Disease Caused by *P. aristosporum*.

(A) *opr7-5 opr8-2* plants germinated until the V2 stage in sterile soil and then transplanted to the field are blighted 6 d later. WT, the wild type.

(B) Rescue of susceptibility of *opr7-5 opr8-2* to *P. aristosporum* by drenching the rhizosphere with 100 mL of 500  $\mu$ M MeJA every 2 d.

(C) Survival rate of plants transplanted in the field as in (A). Error bars represent SD.

(D) The roots of *opr7-5 opr8-2* plants grown in nonsterile soil under greenhouse conditions; *Pythium* oospores were observed in the necrotic root tissue.

(E) The roots of *opr7-5 opr8-2* grown in nonsterile soil following rhizosphere treatment with 100 mL of 500  $\mu$ M MeJA every 2 d.

(F) and (G) A *Pythium* spore suspension was applied to the rhizosphere (see Methods) of the seedlings of the following genotypes: the wild type, *opr7-5*, *opr8-2*, and *opr7-5 opr8-2* (F) and double mutant combinations *opr7-5 opr8-3* and *opr7-5 opr8-4* (G).

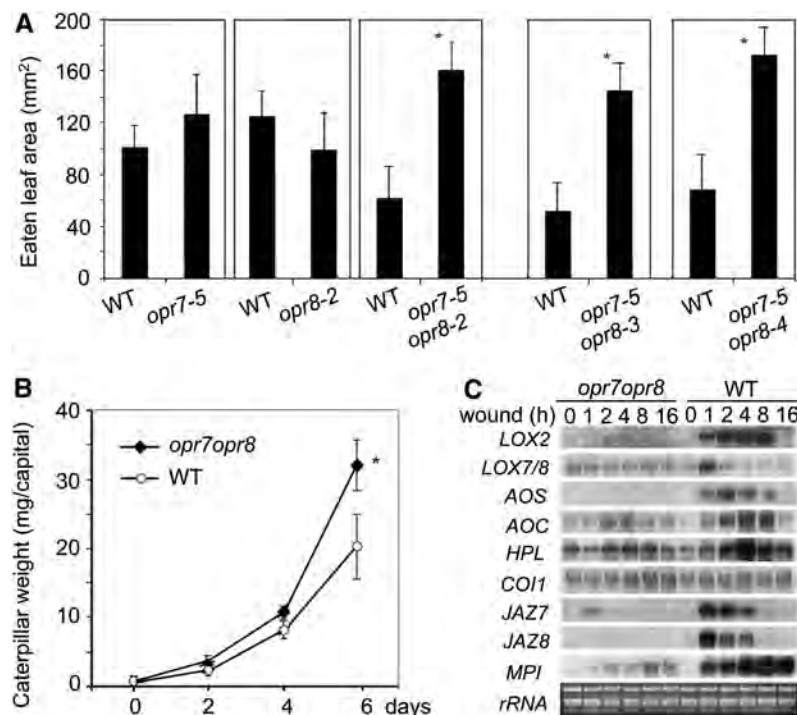
ability of maize roots against *Pythium* species by regulating the expression of multiple JA-dependent defense genes in roots.

### *opr7 opr8* Mutants Are Compromised in Resistance to a Chewing Herbivore

To assess the role of JA in direct defenses against herbivorous insects, a choice-feeding experiment with beet armyworm (BAW; *Spodoptera exigua*) was performed. The leaves of *opr7-5*, *opr8-2*, *opr7-5 opr8-2*, *opr7-5 opr8-3*, and *opr7-5 opr8-4* were placed together with wild-type leaves, into different insect cages (3.5 cm in diameter) and challenged with two 3rd-instar *S. exigua* larvae for 24 h. Figure 8A shows that the single mutants (*opr7-5* or *opr8-2*) were comparable to the wild type in larval preference, but the double mutant combinations *opr7-5 opr8-2*, *opr7-5 opr8-3*, and *opr7-5 opr8-4* were all preferred significantly more than wild-type plants. To test further for the function of JA in insect defense responses, *opr7-5 opr8-2* and wild-type plants were individually caged and infested with two 1st-instar larvae. Larval weight gain was taken at 0, 2, 4, and 6 d after infestation. Figure 8B shows that larvae fed on *opr7-5 opr8-2* gained significantly more weight than those that fed on the wild type. These results suggest that *opr7 opr8* is more susceptible to BAW than either single mutants or the wild type, indicating that

the two JA biosynthesis genes are redundant and required for resistance to herbivorous insects in maize.

A number of studies have shown that wounding of leaves mimics insect feeding and activates similar herbivory defense response pathways in plants (Green and Ryan, 1972; Reymond et al., 2004; Chung et al., 2008). To understand the molecular mechanisms underlying the increased susceptibility of *opr7 opr8* to insect herbivory, we used DNA gel blots probed with cDNA (Zou et al., 2010), so-called reverse RNA gel blots or macroarrays, to monitor the expression of a select set of defense genes in *opr7 opr8* and the wild type in response to mechanical wounding. The macroarray contained 86 probes (gene IDs and primers are listed in Supplemental Table 1 online) representing 85 JA-related or defense-responsive genes. This selection included the entire gene families of maize *LOX* (see Supplemental Table 1 online), *OPR* (Zhang et al., 2005), and *JAZ* genes (maize *JAZs*, see Supplemental Table 1 online; *Arabidopsis JAZs*, Yan et al., 2007). We also selected other defense genes of interest, including *MPI* (Tamayo et al., 2000), the ethylene biosynthesis genes *ACS2*, *ACS6*, and *ACS7* (Young et al., 2004), the insect defense gene *Mir1* (Ankala et al., 2009), the defensin gene *PDC* (Kant et al., 2009), and the anthocyanin biosynthesis genes *A1*, *A2*, *C2*, *BZ1*, and *BZ2* (see Supplemental Table 1 online). The macroarray profile revealed that 67 of this 85-gene set, including *LOX7/8*, *LOX9*, *AOS1*, *OPR7/8*, *MYC2*, *JAZ7*, *JAZ8*, and *MYC2*, etc., were upregulated 1 and 2 h



**Figure 8.** Double Mutant *opr7 opr8* Is Susceptible to BAW (*S. exigua*) Feeding.

**(A)** Choice-feeding by BAW larvae. Each cage contained a wild-type (WT) and a mutant leaf and two 3rd-instar larvae that fed for 24 h. Leaves were scanned and eaten area calculated with ImageJ.

**(B)** Larval growth from infested *opr7-5 opr8-2* and wild-type plants over a 6-d time course.

**(C)** RNA gel blot analysis of expression of JA-dependent genes (see gene IDs in Supplemental Table 1 online) in response to wounding of *opr7-5 opr8-2* and wild-type plants. The V2 stage leaves were wounded at 1-cm intervals with blunt forceps. Error bars represent sd. Significance at \*P ≤ 0.05/\*\*P ≤ 0.01.

after wounding in the wild type but were highly reduced in the double mutant *opr7-5 opr8-2* (see Supplemental Table 2 online). Interestingly, three of these genes, *OPR1*, *OPR2*, and *JAZ9*, were still wound inducible in *opr7-5 opr8-2*, although at a much lower rate than in the wild type (see Supplemental Table 2 online). Our data showed that most of the selected wound-inducible genes in maize are JA dependent. To confirm the macroarray results and test expression of selected genes at additional time points after wounding, we performed RNA gel blot analysis of some of these JA-dependent genes. JA biosynthesis genes (e.g., *AOS* and *AOC*), JA signaling genes (e.g., *JAZ7* and *JAZ8*), and defense genes (e.g., *MP1*) had much lower expression levels in *opr7-5 opr8-2* compared with the wild type (Figure 8C) at all time points tested. *LOX7/8* transcripts appeared to accumulate to slightly higher levels constitutively in *opr7 opr8*; however, no induction of expression was observed in response to wounding normally seen at 1 h after wounding in the wild type. Taken together, our results demonstrate that the JA signaling network in *opr7 opr8* double mutants was attenuated due to JA deficiency.

## DISCUSSION

To date, physiological functions of diverse members of plant *OPR* gene families remain poorly understood in dicotyledonous plant species, let alone monocots. Our long-term goal is to define the roles of all eight members of the maize *OPR* gene family through a transposon-insertional mutagenesis approach. In this study, we created near-isogenic knockout mutants and wild-type lines for the two closely related segmentally duplicated paralogs *OPR7* and *OPR8*, belonging to the putative JA-producing OPRII gene subfamily of maize (Zhang et al., 2005). The two genes share 94.5% amino acid sequence identity but are located on separate chromosomes that presumably evolved as a result of an ancient genome duplication event (Zhang et al., 2005). The generation of the knockout mutants has allowed us to unravel both gene-specific and redundant biochemical and physiological functions of these two genes. As predicted, because these genes encode the only OPRII isoforms in maize, *opr7 opr8* double mutants are defective in JA biosynthesis in all the maize organs tested; no JA was detectable in young leaves (at V2 stage) and roots, and significantly reduced levels were found in the adult plant leaves and in 1- to 2-cm-long developing tassel. This global and strong reduction of JA resulted in multiple and exceptionally penetrant phenotypes and showed that JA functions in sex determination of tassel primordia, normal female organ outgrowth, immunity against soil-borne pathogens and insects, regulation of senescence, and production of anthocyanin pigments.

### Both *OPR7* and *OPR8* Are Redundant for JA Biosynthesis in Most Tissues

Most plants in which JA-producing OPRs were functionally characterized contain only a single member of the OPRII gene subfamily. By contrast, the maize OPRII subfamily contains two closely related paralogs (Zhang et al., 2005). It is generally believed that individual genes exert their functions via differential organ- and stress-specific expression. Interestingly, *OPR7* and *OPR8* were expressed in all tissues tested, although their

expression levels were different (Figure 2A). In most tissues and treatments, both genes appeared redundant in JA biosynthesis, as JA levels were significantly reduced in *opr7 opr8* double mutants only. The two exceptions are JA levels in roots at V4 stage and leaves of adult V12 plant, where disruption of *OPR8* alone resulted in significant reduction of JA, suggesting a unique role of *OPR8* in JA synthesis in these two organs. In leaves of young seedlings, we found *OPR7* to be wound responsive, unlike *OPR8*, which is constitutively expressed, suggesting a degree of functional specialization in response to mechanical damage (Figure 2B). However, despite the fact that only *OPR7* is wound inducible, wound-induced JA levels were not reduced in either the *opr7-5* single mutant or in any of the *opr8* single mutants but only in the double mutant. These results indicate that OPR activity (or enzyme availability) is not rate limiting in wound-induced JA biosynthesis in the single mutants. Another interesting observation is that *opr7 opr8* produced low but measurable levels of JA (approximately one-quarter of the wild type) in leaves but not in tassels and roots (Figures 2D to 2F). This indicates an intriguing possibility that some members of the six-member OPR subfamily are capable of producing JA in leaves, at least in the absence of *OPR7* and *OPR8*. Construction and characterization of other OPR gene mutants, such as *opr1*, *opr2*, *opr5*, and *opr6*, are underway in our current research program and should help illuminate such a hypothetical role of any OPR enzymes in JA biosynthesis in older leaves.

### JA Is Required for Male Sex Determination

JA quantification showed that, unlike single mutations, disruption of both genes resulted in systematic JA depletion in reproductive organs. The most obvious phenotype observed on *opr7 opr8* is tasselseed. Consistent with the role of JA in male organ formation, all three null combinations, *opr7-5 opr8-2*, *opr7-5 opr8-3*, and *opr7-5 opr8-4*, displayed the tasselseed phenotype (Figures 3A and 3B). *OPR7* and *OPR8* are functionally redundant for normal tassel development, since single mutants *opr7* (*opr7-1\**, *opr7-2\**, *opr7-3\**, and *opr7-5*) and *opr8* (*opr8-2*, *opr8-3*, and *opr8-4*) and all weak *OPR7* alleles in combination with all *OPR8* alleles (i.e., *opr7-1\*opr8-2*, *opr7-2\*opr8-2*, and *opr7-3\*opr8-2*) had normal tassel morphology (Figure 3B). Exogenous application of JA into the whorl of *opr7 opr8* reversed the feminized tassel into a normal male tassel (Figure 3A), confirming that JA is required for male inflorescence development. Six *ts* maize mutants have been well characterized, and the genes responsible for four of them, *ts1* (Acosta et al., 2009), *ts2* (DeLong et al., 1993), *ts4* (Chuck et al., 2007), and *ts6* (Chuck et al., 2007), have been cloned. Showing JA deficiency is responsible for the tasselseed phenotype, our results are in accordance with the recent report that *ts1* is due to a mutation of a JA-producing lipoxygenase (Acosta et al., 2009). Collectively, *opr7 opr8* and *ts1* mutant analyses provide strong support for the notion that JA is a signal required for male sex determination in monoecious plants, such as maize.

Unlike maize *ts1* and *opr7 opr8* mutants, which are incapable of forming male inflorescence, all *Arabidopsis* JA-deficient mutants, such as *opr3*, *aos*, the triple mutant *fad3/7/8*, and the JA signaling mutant *coi1*, form flowers containing stamen, but all these mutants are male sterile for three reasons: (1) the anther locules do

not dehisce at flowering time, (2) anther filaments do not elongate sufficiently to position the anther at the appropriate height of the stigma at the time of flower opening, and (3) the pollen grains are predominantly (>97%) inviable (Browse, 2009). In tomato, the JA signaling mutant *jai1-1* is male fertile, but the pollen viability and germination rate are significantly reduced (Li et al., 2004). These results indicated that JA has a role in pollen maturation in both dicotyledonous species. Comparing the studies in dicotyledonous plants and in maize, we conclude that the effects of JA on male sex determination and fertility are not conserved between maize and the two dicotyledonous species studied to date.

### ***opr7 opr8* Mutants Reveal a Novel JA Function in Controlling Outgrowth and Elongation of Female Shoots**

Unlike *ts1* and any other tasselseed mutants, *opr7 opr8* produced multiple ear buds and three to four of these were extremely elongated (Figures 4B to 4D). This remarkable phenotype was especially evident when all the leaves of the *opr7 opr8* V12 stage plants (before tassel emergence) were stripped from the stem, thus revealing that each nodal position has an elongated ear bud, several of which developed into abnormally elongated ear shoots (Figures 4F to 4H). This phenotype of the double mutants is clearly mediated by JA signaling, as exogenous JA application complemented the wild-type morphology, both in the appearance of multiple ear shoots and abnormal elongation of ear shanks (Figure 4I). Our results illustrate that *opr7 opr8* lost the ability to inhibit the outgrowth of ear buds and ear shoot elongation, suggesting that JA is necessary in maize to inhibit both elongation of axillary buds and elongation of ear shanks. Although the precise molecular mechanism behind the formation of multiple elongated ears and axillary buds in *opr7 opr8* is not known, one potential mechanistic explanation may be that the lack of JA may alter biosynthesis or transportation of auxin or/and strigolactone, the two hormones implicated in the regulation of axillary bud elongation in plants (Leyser, 2009). Supporting such a possibility, we showed that in the senescing leaves JA signal affects ethylene, ABA, and CK biosynthesis (Figures 5D to 5F).

Information on the role of JA in female organ development or fertility is limited, even in dicotyledonous plants, except for the tomato JA signaling mutant *jai1-1*. This mutant exhibited severely decreased female fertility (<1%) caused by a defect in the maternal control of seed embryo growth, which was associated with the loss of accumulation of JA-regulated proteinase inhibitor proteins in fruit tissues (Li et al., 2004).

### **JA Is an Important Signal Facilitating Leaf Senescence in Maize**

One of the strong phenotypes consistently observed in *opr7 opr8* double mutants is the delay of leaf senescence (Figures 5A to 5C), indicating that JA plays an important role in leaf aging. Although JA has long been implicated as a senescence-promoting hormone (Ueda and Kato, 1980; He et al., 2002; Schenk et al., 2000; Castillo and León, 2008), recent studies have brought the role of JA in leaf senescence into question. The JA-defective *Arabidopsis* mutants *aos* and *opr3* did not show altered senescence phenotypes during natural senescence processes or upon dark

treatment (He et al., 2002; Schommer et al., 2008). In addition, chlorophyll loss in the leaves during natural senescence and upon dark incubation was not altered in LOX2-RNA interference plants, which exhibited no increased accumulation of JA or OPDA (Seltmann et al., 2010). This uncertainty led us to test whether JA deficiency in *opr7 opr8* mutants may have altered the content of other plant hormones reported to have a strong association with leaf senescence. With this idea, we tested levels of ethylene, ABA, and CK in the oldest two leaves undergoing senescence. A modest ~25% reduction in ethylene may partially explain the delayed senescence phenotype in *opr7 opr8* leaves (Figure 5D). By contrast, ABA content in senescing double mutant leaves was fourfold lower than the wild type (Figure 5E), indicating that ABA may be the major reason for the delayed leaf senescence in the double mutants. ABA is a known positive regulator of leaf senescence (Lim et al., 2007). Considering that CKs play a positive regulatory role in growth and a negative role in leaf senescence (Riefler et al., 2006), we hypothesized that *opr7 opr8* has delayed leaf senescence because of high accumulation of CKs in the leaves due to JA depletion. Contrary to our hypothesis, *opr7 opr8* was found to produce much less CKs than wild-type plants (Figure 5F; see Supplemental Figure 5 online). These data suggest that JA is a positive regulator of CK production in maize leaves and that the delayed leaf senescence phenotype of *opr7 opr8* is not relevant to CK levels.

### **Anthocyanin Pigmentation in Brace Roots Requires JA**

The regulation of anthocyanin biosynthesis has long been a broad interest to maize geneticists (Dooner et al., 1991; Bruce et al., 2000), yielding a good understanding of the major enzymes and transcription factors involved (Sharma et al., 2011). Because JA mutants have not been available, it is not surprising that JA has not been implicated in the regulation of anthocyanin biosynthesis in monocots. However, a number of studies have shown that JA induces anthocyanin accumulation in several dicotyledonous plants (Shan et al., 2009). JA-regulated pigmentation in *Arabidopsis* was shown to be dependent on JA signaling SCF-COI1 complex interacting with WD-repeat/bHLH/MYB complexes (Qi et al., 2011). However, even in the dicotyledonous species, JA dependency of anthocyanin biosynthesis is not thoroughly investigated in an organ-specific manner. In this study, we investigated the tissue-specific JA dependency of anthocyanin pigmentation in maize. We found that *opr7 opr8* visibly lacks anthocyanin production in the leaf auricles (Figure 6B) and brace roots (Figure 6A), a phenotype that is readily reversed to the wild type by exogenous JA. These data provide strong genetic evidence for the link between JA signaling and anthocyanin accumulation in maize. Moreover, transcriptional profiling of anthocyanin biosynthesis pathway genes in the brace roots of *opr7 opr8* and the wild type revealed that two of these genes, *F3H* and *A1*, are likely targets for the JA-mediated regulation of this pathway (Figure 6). Taken together, our results demonstrated that in maize anthocyanin pigmentation is dependent on JA signaling in some tissues, such as crown roots and leaf auricles.

### **JA Is Essential for Maize Plant Survival in Nature**

Here, we found that the double mutant *opr7 opr8* is not viable under natural field conditions or in nonsterile potting soil. By contrast,

single mutant *opr7* or *opr8* plants grew well and developed like wild-type plants. JA analysis showed that *opr7* had normal levels of JA in roots, *opr8* produced lower levels of JA, and no JA was detectable in the double mutant roots. Since even significantly reduced levels of JA in the *opr8* roots allowed seedling to overcome damping-off disease, we conclude that even these low JA levels are sufficient to provide resistance against soil-borne pathogens. However, JA deficiency results in complete lack of innate immunity against soil-borne root-rotting pathogens. The death of double mutants in the field due to pre- and postgermination damping-off disease was diagnosed to be caused by the necrotrophic root-rotting oomycete *Pythium* spp. In the greenhouse experiments, we found that *P. aristosporum* was present in the artificial soil we used (SB300; Sun Gro). This pathogen proved to be lethal for *opr7 opr8* but not single mutant or wild-type plants. *opr7 opr8* mutants complemented by exogenous JA were as resistant to *P. aristosporum* as the wild type, thus demonstrating that the extreme susceptibility of the double mutant to *Pythium* spp was due to JA deficiency. In *Arabidopsis*, JA biosynthesis mutant *fad3/7/8* and signaling mutant *jar1* displayed increased susceptibility to the necrotrophic pathogens, *Botrytis* spp and *Alternaria brassicicola* (Van Baaren et al., 2007; Pré et al., 2008), *Pythium mastophorum*, and *Pythium irregulare* (Staswick et al., 1998; Vijayan et al., 1998). Combining our results in maize with these studies of *Arabidopsis*, we can clearly conclude that JA-mediated responses drive a conserved mechanism for plant immunity against damping-off disease caused by the soil-borne oomycete *Pythium* spp.

### JA Is an Indispensable Signal in Maize for Defense Response against Herbivores

Although JA-regulated defense pathways have been extensively studied in the model plant *Arabidopsis* and other dicots (Howe and Jander, 2008), far less is known about their role in regulating direct herbivore defenses in monocots, especially in an economically significant crop like maize (Ankala et al., 2009). The creation of JA-deficient *opr7 opr8* mutants provided an excellent tool to test whether JA has a similar insect defense signaling role in maize. Our data unanimously showed that all three *opr7 opr8* double mutant combinations were significantly preferred by BAW larvae in choice experiments. Furthermore, larvae feeding on *opr7 opr8* grew much faster than on the wild type (Figures 6B and 8A), demonstrating that JA deficiency significantly reduces resistance of maize against the chewing herbivore BAW. These results provide strong genetic support for previous pharmacological evidence demonstrating that induction of JA is one of the major signaling events in maize responsible for the activation of direct and indirect defenses against insect herbivory (Engelberth et al., 2004, 2007).

The lack of JA-deficient mutants has significantly impeded the progress of maize geneticists interested in studying the relevance of JA in maize adaptation to multiple environmental stresses. For example, a strong need exists to identify marker genes that are strictly JA dependent. Moreover, the maize genes for the JA signaling pathway are largely unknown, let alone those that are responsive to insect attack. To fill in this gap in our knowledge, we thoroughly searched the maize genome databases (MaizeGDB, The TIGR Maize Database, and PlantGDB) for the orthologs of JA biosynthesis and signaling genes in *Arabidopsis* and rice. The

closest orthologous genes of *AOS*, *AOC*, *COI1*, *MYC2*, *JAR1*, *ERFs*, and *JAZs* in the maize genome were found (see Supplemental Table 1 online). A macroarray for transcript profiling was constructed that included gene-specific probes for these orthologs and the entire gene families of *LOXs*, *OPRs*, *JAZs*, and 28 other defense genes. The following genes were found to be strictly JA dependent and are excellent candidates for JA-mediated defense markers: *LOX7/8*, *LOX9*, *AOS1*, *OPR7/8*, *MYC2*, *JAZ7*, *JAZ8*, and others (see Supplemental Table 2 online). *OPR1*, *OPR2*, *JAZ9*, and *HPL* were also reduced in *opr7 opr8* mutants but still showed wound inducibility, suggesting additional signals regulate their wound responsiveness. In summary, these macroarray and RNA gel blot analyses elucidated a gene set regulated by JA and further demonstrated that the maize JA biosynthesis and signaling pathway requires JA for positive feedback loop regulation. Also, similar to *Arabidopsis* and other dicots (Howe and Jander, 2008), these pathways appear to be indispensable in maize defense ability against insect herbivory.

## METHODS

### Identification of *Mu* Insertions of *opr7* and *opr8* Alleles and Construction of Near-Isogenic Mutant and Wild-Type Lines

The insertions in *OPR7* and *OPR8* were isolated by PCR genotyping at Pioneer-a DuPont Company, which possesses a library of transposon-mutagenized seeds (McCarty and Meeley, 2009) comprising a large *Mu*-inserted population of ~42,000 individuals. *Mu* insertions in *OPR7* or *OPR8* were identified by PCR genotyping as described by Bomblies et al. (2003) using the *Mu* terminal inverted repeat primer 9242 (all the primers used in this study are listed in Supplemental Table 1 online) in combination with *OPR7*-specific primers 80544 or 80545 or *OPR8*-specific primer 80548. Four *Mu*-mutagenized alleles (*opr7-1*, *7-2*, *7-3*, and *7-4*) of *OPR7* and four alleles (*opr8-1*, *8-2*, *8-3*, and *8-4*) of *OPR8* were identified. To eliminate extraneous *Mu* transposons, we backcrossed the original allele materials to the inbred line B73 more than five times. During genetic advancement, mutant alleles were identified by PCR using the gene-specific primers *OPR7F* and *OPR7Rin* or *OPR8F* and *OPR8Rs*. While genotyping, we unexpectedly found a new allele (*opr7-5*) derived from transposase activity, which moved the *Mu* transposon from the *opr7-3* site to the first exon of *OPR7* (see Supplemental Figure 3 online). RNA gel blot and RT-PCR analyses were used to measure transcript accumulation in the alleles of *OPR7* and *OPR8*. To investigate functional redundancy between *OPR7* and *OPR8*, we generated *OPR7* and *OPR8* double mutants by crossing *opr7-5* with *opr8-2* at the BC5 stage to produce the *opr7-5 opr8-2* double mutant. As different negative and positive controls, alternative cross combinations between *opr7* and *opr8* mutant alleles were also performed (i.e., *opr7-5* × *opr8-3*, *opr7-5* × *opr8-4*, *opr7-1* × *opr8-2*, *opr7-2* × *opr8-2*, and *opr7-3* × *opr8-2*).

### Plant Growth Conditions in Lab and Greenhouse

For laboratory experiments, plants were grown at 25°C, 50% RH, with 14 h of light at 100 mmol m<sup>-2</sup> s<sup>-1</sup>. For seed propagation and greenhouse experiments, plants were grown at 25 to 30°C and 50 to 70% RH with 14 h of natural light plus artificial illumination. The artificial soil mixes, namely, Sunshine SB300 mix and Metro-mix 200 (Sun Gro), were used for all experiments.

### JA Analysis and Chemical Treatment of Tasselseed Plants

For wound-induced JA and OPDA analysis, the second leaf of V-2 stage *opr7*, *opr8*, *opr7 opr8* (see maize [*Zea mays*] growth stages at <http://>

maizedoctor.cimmyt.org/en/getting-started/9?task=view), and wild-type plants were wounded with blunt forceps, and JA and OPDA were measured by gas chromatography–mass spectrometry or liquid chromatography–mass spectrometry (LC-MS) (Engelberth et al., 2003; Koo et al., 2009). For analysis of JA in belowground tissues, the roots of V-4 stage *opr7*, *opr8*, *opr7 opr8*, and wild-type plants were harvested and JA was measured by LC-MS as described by Koo et al. (2009). To measure JA levels in the leaves and tassels of preflowering plants, leaf samples were collected from the middle section of the 13th leaf of plants with 10 fully extended leaves, and the young tassels (4 to 6 cm long) were also harvested from these plants to determine JA levels in male sex organs.

To rescue the *opr7 opr8* tasselseed phenotype, one mL of 1 mM JA (Sigma-Aldrich) was applied to the apical whorl of the mutant as described by Acosta et al. (2009) with a minor modification - the plants were treated eight times instead of three times at an interval of 48 h until the tassel emerged from the whorl. Control plants were treated with 1 mL of 0.005% ethanol, the solvent used for the 1 mM JA solution.

### Other Hormone Measurements

Measurement of ethylene production in the leaves was measured by gas chromatography as described by Gao et al. (2008). Extraction of ABA and CK was modified from Quesnelle and Emery (2007) and Ross et al. (2004) using additional labeled internal standards for CKs, including  $^2\text{H}_4\text{BA}$ ,  $^2\text{H}_7\text{BAR}$ ,  $^2\text{H}_5\text{ZOG}$ ,  $^2\text{H}_5\text{DHZOG}$ ,  $^2\text{H}_5\text{ZROG}$ ,  $^2\text{H}_5\text{DHZROG}$ ,  $^2\text{H}_6\text{iP7G}$ ,  $^2\text{H}_5\text{Z9G}$ ,  $^2\text{H}_5\text{MeSZ}$ ,  $^2\text{H}_6\text{MeSiP}$ ,  $^2\text{H}_5\text{MeZR}$ ,  $^2\text{H}_5\text{MeSiPR}$ ,  $^2\text{H}_6\text{iPP}$ ,  $^2\text{H}_3\text{DHZR}$ ,  $^2\text{H}_6\text{iP}$ ,  $^2\text{H}_3\text{DHZ}$ ,  $^2\text{H}_6\text{Z}$ ,  $^2\text{H}_6\text{iPMP}$ ,  $^2\text{H}_6\text{ZRMP}$ , and  $^2\text{H}_6\text{DHZRMP}$  (OChemIm) and  $^2\text{H}_4\text{ABA}$  (PBI). ABA and CKs were analyzed by the isotope dilution method using HPLC–electrospray ionization tandem mass spectrometry (Dionex HPLC machine connected to a Sciex Applied Biosystems 5500 API mass spectrometer).

### Root Infection with *Pythium* spp

To produce inoculum for root infections, an isolate of *Pythium aristosporum* was grown on 9-cm-diameter Petri dishes containing V8 agar for 4 weeks. The agar was then macerated, suspended in 200 mL of sterile water, and passed through two layers of cheesecloth. Oospore concentrations were determined by hemocytometer and adjusted to  $10^5$  spores/mL. V-2 to V-3 stage plants grown in sandy soil were inoculated by adding 5 mL of suspension per plant to the soil around the roots. The disease symptoms were scored 4 to 6 d after inoculation.

### Insect-Feeding Assay of Mutants

To evaluate insect resistance in *opr* mutants, we infested the third leaf of *opr7-5*, *opr8-2*, *opr7-5 opr8-2*, *opr7-5 opr8-3*, *opr7-5 opr8-4*, and wild-type plants with BAW larvae. For choice assays, mutant and wild-type leaves of the mentioned genotypes were confined to individual cages (3.5-cm diameter), each with one mutant leaf and one wild-type leaf (i.e., *opr7-5* versus the wild type, *opr8-2* versus the wild type, *opr7-5 opr8-2* versus the wild type, *opr7-5 opr8-3* versus the wild type, and *opr7-5 opr8-4* versus the wild type). Two 3rd-instar larvae were placed on the inside bottom of each cage. The cages were moved distally to the uneaten part of the leaf every 12 h. Twenty-four h after infestation, leaf segments were scanned and damaged areas calculated with ImageJ (<http://rsbweb.nih.gov/ij/>). For larval feeding assays, the third leaf of individual genotypes was confined to a 9-cm cage and infested with two 1st-instar larvae. Every 12 h, the cages were carefully moved distally to fresh tissue. At different anticipated time points the larvae were removed from the plants and weighed.

### DNA Gel Blot Analysis

Genomic DNA was isolated from the leaves of 2-week-old plants using a urea-based extraction protocol ([www.Arabidopsis.org/comguide/chap\\_3\\_dna\\_techniques/2\\_DNA\\_extraction.html](http://www.Arabidopsis.org/comguide/chap_3_dna_techniques/2_DNA_extraction.html)). Genomic DNA (15  $\mu\text{g}$ ) was digested with *EcoRI* + *BamHI* (for *OPR7* alleles) or *EcoRV* (for *OPR8* alleles) and hybridized with the *OPR7* or *OPR8* probes. The *OPR7* probe is a 199-bp fragment in the middle part of the gene. This probe can hybridize to *OPR7* and *OPR8*; therefore, it yields two bands on the blot. It was amplified from cDNA using *OPR7-425F* and *OPR7-624R* primers. For *OPR8*, the probe is a 204-bp fragment of 3' UTR, which specifically hybridizes to *OPR8*. This probe was amplified from genomic DNA using primers *OPR8-probF* and *OPR8-probR*. The procedure was done according to a DNA gel blot protocol ([http://www.protocol-online.org/cgi-bin/prot/view\\_cache.cgi?ID=2746](http://www.protocol-online.org/cgi-bin/prot/view_cache.cgi?ID=2746)). The probes were labeled using Ready-To-Go DNA labeled beads (GE Healthcare UK Limited) with  $^{32}\text{P}$ -dCTP according to the manufacturer's protocol. Blot membranes were exposed to x-ray film (Kodak) in cassettes at  $-80^\circ\text{C}$  for 3 to 10 d depending on the signal strength.

### RNA Gel Blot and Macroarray Analysis

Gene expression analysis of JA-dependent genes in wounding response was performed by RNA gel blot analysis according to an online protocol (<http://www.protocol-online.org/prot/Protocols/Northern-Hybridization-of-RNA-Fractionated-by-Agarose-formaldehyde-Gel-781.html>) using 10  $\mu\text{g}$  of total RNA. The hybridization probes were labeled with  $^{32}\text{P}$ -dCTP using gene-specific primers (see Supplemental Table 1 online) according to the protocol. For *OPR7*, a 154-bp fragment of the 3' UTR was amplified from cDNA using primers *7Q2F* and *7Q5R* to be used as a probe. This probe preferentially hybridized to *OPR7* but may also weakly hybridize to *OPR8* because 89 bp of the probe shares a high degree of sequence identity with *OPR8*. For *OPR8*, the probe is *OPR8* specific as in the DNA gel blot.

Macroarrays contained 85 gene-specific tags, which were amplified by PCR from genomic DNA using gene-specific primers (see Supplemental Table 1 online). The PCR products were purified using a gel extraction kit (No. 28604; Qiagen) and adjusted to 20 ng/ $\mu\text{L}$  with the elution solution. Purified products were denatured at  $100^\circ\text{C}$  for 1 to 2 min, immediately placed on a nylon membrane (GE Water and Process Technologies), and then incubated at  $70^\circ\text{C}$  for 2 h in preparation for hybridization. mRNA was isolated from the total RNA of the samples using an mRNA mini kit (No. 70022; Qiagen). Five hundred nanograms of mRNA was used for reverse transcription with  $^{32}\text{P}$ -dCTP (Qiu et al., 2004) using the SuperScript III First Strand kit (No. 18080-051; Invitrogen) with an oligo(dT<sub>20</sub>) primer. Subsequent steps, including hybridization, membrane washing, and imaging, are identical to the RNA gel blot procedure. The macroarray images on the x-ray film were scanned, and the mean intensity of each spot was calculated using ImageJ (<http://rsbweb.nih.gov/ij/>).

### Real-Time PCR and RT-PCR

RNA extraction and cDNA synthesis were conducted using a TRI Reagent kit (Molecular Research Center) and the First-Strand cDNA synthesis kit (Amersham Biosciences UK Limited), respectively, according to the manufacturer's protocol. Real-time PCR was run using a SYBR green RT-PCR kit (No. 204243; Qiagen) with 25- $\mu\text{L}$  reactions prepared with a final primer concentration of 0.5  $\mu\text{M}$ . The amplification was performed using the 7500 Real-Time PCR system (Applied Biosystems) with the following parameters: 15 min at  $95^\circ\text{C}$ ; 35 s at  $94^\circ\text{C}$ , 35 s at  $53^\circ\text{C}$ , and 15 s at  $72^\circ\text{C}$ , with 40 cycles; and 10 min at  $72^\circ\text{C}$ . Maize glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) was used as reference gene. The amplification data analysis was done according to the manufacturer's protocol.

For RT-PCR, total RNA was treated with RNase-free rDNase at  $37^\circ\text{C}$  for 30 min using the DNA-free kit (Ambion) to eliminate trace amounts of genomic DNA in the RNA samples. cDNA was synthesized using the First-

Strand cDNA synthesis kit (GE Healthcare) with oligo(dT)<sub>18</sub> primers following the manufacturer's protocol. A Taq PCR Core kit (Qiagen) was used to amplify the interest genes using cDNA as template with amount equal to 50 ng of total RNA for one 25- $\mu$ L reaction. The amplification was conducted in a VERITI thermal cycler with the following thermal cycler conditions: 10 min at 94°C; 45 s at 94°C, 45 s at 56°C, and 1 min at 72°C, with 32 cycles; and 10 min at 72°C. *GAPDH* was used as reference. Amplified fragments were separated on 1% (w/v) agarose gels containing 0.5  $\mu$ g/mL ethidium bromide, and images were visualized by the Alpha Imager 2000 software (Alpha Innotech).

### Statistical Analysis of Data

A Student's *t* test was performed between the wild type and mutant for JA content (Figure 2), leaf damage (Figure 8), leaf senescence (Figure 5), and gene expression for the study of anthocyanin pigmentation (Figure 6). Four individual plants as four biological replicates were analyzed for each genotype. Significant differences were  $P \leq 0.05/P \leq 0.01$ . The error bars in the figures represent sd.

### Accession Numbers

Sequence data for *OPR7* and *OPR8* can be found in the GenBank/EMBL data libraries under accession numbers AY921644 and AY921645, respectively. The GenBank accession numbers for the genes used for microarray profiling or RNA gel blot analysis are listed in Supplemental Table 1 online.

### Supplemental Data

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

**Supplemental Figure 1.** The Insertion Sites of *Mutator* Transposons in the *opr7* Alleles.

**Supplemental Figure 2.** The Insertion Sites of *Mutator* Transposons in the *opr8* Alleles.

**Supplemental Figure 3.** Sequencing *Mu* Insertion Altered Transcript of *OPR7* in the *opr7-5* Allele.

**Supplemental Figure 4.** The Close-Up Comparison of the Morphology of the Axillary Buds between *opr7 opr8* and the Wild Type.

**Supplemental Figure 5.** Content of Cytokinins in First and Second Leaves of Double Mutant *opr7-5 opr8-2* and the Wild Type at Different Leaf Ages.

**Supplemental Figure 6.** *opr7 opr8* Double Mutants Are Not Viable under the Field Conditions.

**Supplemental Figure 7.** *opr7 opr8* Is Susceptible to *Pythium* spp Present in the Artificial Soil Mixes Used, and the Phenotype Is Reversed to Wild-Type by MeJA Application.

**Supplemental Figure 8.** Expression of JA-Dependent Genes or Defense Genes in the Roots in Response to *Pythium aristosporum* Inoculation.

**Supplemental Table 1.** Primers Used in This Study.

**Supplemental Table 2.** Expression of Wound-Inducible Genes in the Leaves of *opr7 opr8* Mutant and the Wild Type 1 or 2 h after Wounding.

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### AUTHOR CONTRIBUTIONS

Y.Y. performed the majority of the experiments and drafted the article. S.C. performed JA analysis by LC-MS, helped with resistance assay to BAW, and revised the draft. T.I. identified the causal agent of damping-off and assisted with inoculation experiments. J.E. performed quantification of octadecanoids by gas chromatography-mass spectrometry. R.M. identified the *Mu* insertional alleles of *OPR7* and *OPR8*. A.H. and R.J.N.E. performed ABA and CK measurement. M.V.K. directed this study, participated in the experiments, and revised the article. All the authors read and approved the final article.

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**Disruption of *OPR7* and *OPR8* Reveals the Versatile Functions of Jasmonic Acid in Maize Development and Defense**

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