Running Title: Peptide-Regulated Maize Defense

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Title: ZmPep1, an Ortholog of Arabidopsis Elicitor Peptide 1, Regulates Maize Innate Immunity and Enhances Disease Resistance

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Abstract

ZmPep1 is a bioactive peptide encoded by a previously uncharacterized Zea mays gene, ZmPROPEP1. ZmPROPEP1 was identified by sequence similarity as an ortholog of the Arabidopsis AtPROPEP1 gene, which encodes the precursor protein of elicitor peptide 1 (AtPep1). Together with its receptors, AtPEPR1 and AtPEPR2, AtPep1 functions to activate and amplify innate immune responses in Arabidopsis, and enhances resistance to both Pythium irregulare and Pseudomonas syringae. Candidate orthologs to the AtPROPEP1 gene have been identified from a variety of crop species, however, prior to this study, activities of the respective peptides encoded by these orthologs was unknown. Expression of the ZmPROPEP1 gene is induced by fungal infection and treatment with jasmonic acid (JA) or ZmPep1. ZmPep1 activates de novo synthesis of the hormones JA and ethylene (ET) and induces expression of genes encoding the defense proteins Endochitinase A, PR-4, PRms and SerPIN. ZmPep1 also stimulates expression of BX1, a gene required for biosynthesis of benzoazinoid defenses, and accumulation of 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) in leaves. To ascertain whether ZmPep1-induced defenses affect resistance, maize plants were pretreated with the peptide prior to infection with fungal pathogens. Based on cell death and lesion severity, ZmPep1 pretreatment was found to enhance resistance to both southern leaf blight and anthracnose stalk rot caused by Cochliobolis heterostrophus and Colletotrichum graminicola respectively. We present evidence that peptides belonging to the elicitor peptide (Pep) family have conserved function across plant species as endogenous regulators of innate immunity and may have potential for enhancing disease resistance in crops.
Introduction

Peptides regulate diverse processes pertaining to both development and defense in plants (Matsubayashi and Sakagami, 2006). Defensively, peptides can act as molecular messengers during plant interactions with other organisms, alerting the plant to potential attack and inducing defenses. Microbe-associated molecular patterns (MAMPs) are molecular fragments recognized by plants as indicators of potential invasion, and peptide MAMPs derived from microbial proteins, such as flg22, elf18 and Pep13, are bound by specific plant pattern-recognition receptors to elicit a cascade of downstream defense responses (Hahlbrock et al., 1995; Zipfel et al., 2004; Zipfel et al., 2006). Peptides also warn plants of attack by insect herbivores; the inceptin peptide is one such herbivory-associated molecular pattern (HAMP) that activates downstream defenses in response to herbivory (Schmelz et al., 2006; Mithöfer and Boland, 2008).

In addition to peptide MAMP elicitors that alert plants to the presence of invading organisms, there are several classes of endogenous plant peptides that regulate defenses, acting as internal elicitors (Ryan et al., 2007). Biotic stress resulting in cellular damage induces expression of the genes encoding endogenous peptide precursor proteins and the activated peptides then contribute to defense through amplification of plant responses. Systemin and hydroxyproline-systemin (HypSys) peptides function as endogenous regulators of defense against herbivores (Ryan and Pearce, 2003; Narváez-Vásquez et al., 2007). Signaling by these peptides promotes a myriad of anti-herbivore responses including accumulation of proteinase inhibitor proteins and of other antinutritive proteins such as polyphenol oxidase, threonine deaminase and arginase as well as systemic emission of volatiles (Pearce et al., 1991; Howe and Jander, 2008; Degenhardt et al., 2010). Other peptides are endogenous regulators of pathogen defense responses; recently, soybean has been discovered to produce a unique peptide signal, GmSubPep, which activates transcription of pathogen defense genes (Pearce et al., 2010). In Arabidopsis, elicitor peptide 1 (AtPep1) belongs to a family of peptides that interact with the PEPR receptors to regulate
expression of pathogen defense genes, including those encoding the PDF1.2 defensin and PR-1 (Huffaker et al., 2006; Yamaguchi et al., 2006; Yamaguchi et al., 2010).

While systemin and AtPep1 are endogenous defense signals as opposed to MAMP/HAMP exogenous elicitors and indicators of non-self, the signaling similarities shared by these peptide regulators closely resembles aspects of MAMP/HAMP-induced signaling (Ryan et al., 2007). AtPep family peptides and peptide MAMPs such as flg22 and elf18 activate similar downstream responses using many of the same molecular components (Ryan et al., 2007; Yamaguchi et al., 2010; Postel et al., 2010; Krol et al., 2010). Both flg22 and AtPeps bind specific LRR receptors, and both activate downstream defense genes through a myriad of downstream second-messenger signals, which in addition to jasmonate and hydrogen peroxide, are believed to include ethylene, salicylate and membrane depolarization (Yamaguchi et al., 2006; Huffaker and Ryan, 2007; Krol et al., 2010). The receptors for both flg22 and AtPep1 associate with an interacting receptor partner, BAK1, and likely activate cyclic nucleotide-gated calcium channels via receptor guanylyl cyclase activity (Postel et al., 2010; Ma et al., 2009). Treatment with flg22 upregulates transcription of genes encoding PROPEP family precursors and both PEPR receptors, and AtPep1-treatment induces transcription of FLS2, the flg22 receptor (Zipfel et al., 2004; Ryan et al., 2007).

The breadth of responses regulated by endogenous peptides indicates their potential utility as a mechanism for the manipulating resistance, a strategy that has been demonstrated through experiments with transgenic plant lines. Solanaceous plants constitutively expressing the genes encoding proSystemin or proHypSys accumulate herbivore defense proteins to much higher levels than wild type plants and are more resistant to insect attack (Bergey et al., 1996; Ren and Lu, 2006). Similarly, Arabidopsis plants constitutively expressing the AtPROPEP1 precursor gene have higher basal expression levels of pathogen defense genes and demonstrate resistance to the necrotrophic pathogen Pythium irregulare (Huffaker et al., 2006). Direct application of peptide to plants is also an effective mechanism to manipulate defense, pretreatment of Arabidopsis plants with either flg22 or AtPep1 peptides prior to inoculation with the
hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) enhanced plant resistance (Yamaguchi *et al.*, 2010; Zipfel *et al.*, 2004).

Enhanced disease resistance obtained through peptide pretreatment or transgenic constitutive expression indicates that such methods could have potential use in the field, especially if the mechanisms are conserved across species. However, systemin is not active in non-Solanaceous plants, nor are AtPep peptides capable of signaling in other species (Ryan and Pearce, 2003; Yamaguchi *et al.*, 2006). This species-specificity has prevented the functional transfer of peptide-enhanced defense to diverse plants species. While a proHypSys ortholog has been identified in *Ipomoea batatas*, indicating that the systemin superfamily does exist in other species, homologs have not yet been identified in any other plant families (Chen *et al.*, 2008). Whether this lack of identified systemin homologs is because related peptides evolved only in the Solanaceae or because the amino acid sequence of functional homologs has diverged to the point of being unrecognizable in other species is unknown.

Orthologs of *AtPROPEP* genes have been identified in other plant species through amino acid sequence comparisons. However, those orthologs share little direct sequence identity to *AtPROPEP* genes. This lack of sequence identity among species is unsurprising, as Arabidopsis peptides that bind the same receptor have precursor amino acid sequence identity between 12 and 47 % (Yamaguchi *et al.*, 2006). All Arabidopsis Pep family precursors do share homologous conserved domains, the combination of which has been used as a means of identification of orthologs in other species. First, all PROPEP family orthologs contain the predicted active peptide sequence at the carboxyl terminus of a larger precursor protein, a characteristic also shared by many animal peptide hormone precursors and by prosystemin (McGurl *et al.*, 1992; Huffaker *et al.*, 2006). None of the precursors have a traditional signal sequence for export through the secretory pathway, but each does encode an amphipathic helix motif that is potentially a site of protein-protein interactions (Rhoads and Friedberg, 1997; Huffaker *et al.*, 2006). All predicted peptides are enriched in basic amino acids and each precursor protein has several repeated “EKE” motifs, consisting of a high density of aspartate/glutamate residues interspersed with lysine/arginine (McGurl *et al.*, 2006).
1992; Realini et al., 1994; Huffaker et al., 2006). None of the genes designated as AtPROPEP orthologs using the above criteria have been studied for functional homology, and it has been suggested that true AtPep1 homologs likely exist only in species closely related to Arabidopsis (Boller and Felix; 2009).

Our studies present evidence that the gene ortholog in Zea mays, ZmPROPEP1, encodes a peptide, ZmPep1, which is an active signal regulating pathogen defense. The ZmPROPEP1 gene is expressed in response to jasmonic acid treatment and fungal infection. Treatment of leaves with ZmPep1 promotes production of the hormones jasmonic acid (JA) and ethylene (ET) and induces expression of genes encoding their biosynthetic enzymes, genes associated with pathogen defense and the ZmPROPEP1 gene. ZmPep1 activates biosynthesis of benzoxazinoid defenses and promotes accumulation of HDMBOA-Glc, a storage form of a highly reactive aglycone hydroxamic acid. Finally, pretreatment with ZmPep1 prior to infection enhances maize resistance to both the foliar pathogen Cochliobolis heterostrophus and the stalk-rot pathogen Colletotrichum graminicola.

Results

Maize transcribes a pathogen-inducible gene orthologous to AtPROPEP1

Using the AtPROPEP1 sequence to query NCBI Zea mays nucleotide sequences, we identified ZmPROPEP1 as a potential homolog. While the amino acid identity between the two precursors is only fourteen percent, both share the modular structural motifs characteristic of the PROPEP family (Figure 1A). These motifs include the amphipathic helix motif that is potentially a site of protein-protein interactions, multiple “EKE” repeats, and location of the active peptides at the carboxyl terminus of both precursors. The native length of is ZmPep1 predicted to be 23 amino acids, as are both AtPep peptides that have been isolated biochemically (Huffaker et al., 2006; Pearce et al., 2008). Neither AtPROPEP1 nor ZmPROPEP1 have a conventional signal sequence for export through the secretory pathway, and both are predicted to localize to the cytosol.

The predicted peptide encoded by the ZmPROPEP1 gene has several conserved residues at the carboxyl end as compared to AtPep1 (Figure 1B), including the glycine
Gly17) shown to be essential for AtPep1 bioactivity (Pearce et al., 2008). Like AtPep1, the amino end of ZmPep1 is enriched in basic residues, and contains five arginines compared to the five lysines and one arginine in the amino region of AtPep1 (Figure 1B). The isoelectric point of both peptides is at very high pH, pI of 11.22 and 12.18 for AtPep1 and ZmPep1 respectively.

The *Zea mays* genomic sequence encoding ZmPROPEP1 was cloned from both var. Golden Queen (GQ), a commercially grown sweet corn, and from B73. As in the Arabidopsis AtPROPEP1 gene, both GQ and B73 genes contained a single short intron just upstream of the encoded peptide (Supplementary Figure 1A). The cloned B73 sequence was found to be identical to database sequences, whereas the GQ gene encoded eight amino acid changes, none of which were in the predicted ZmPep1 peptide (Supplementary Figure 1B). Several cDNAs encoding the ZmPROPEP1 precursor were amplified from young leaf tissue of one month old GQ plants. Sequencing of six independent cDNA clones revealed that three had the intron alternatively spliced such that the transcripts encoded a precursor with five fewer amino acids (Supplementary Figure 1B). This differential splicing could potentially contribute to regulation of peptide processing as the splice site is just upstream of the region encoding ZmPep1, where proteolytic activity likely would release the active peptide from the precursor.

To ascertain whether the ZmPROPEP1 gene responds to pathogen infection, ZmPROPEP1 transcript abundance was analyzed in intact plants that were infected with the fungus *Cochliobolus heterostrophus* versus uninfected control plants. ZmPROPEP1 transcript levels increased in the fungal-infected plants (Figure 1C). Expression of ZmPROPEP1 was also induced in intact leaves treated with either ZmPep1 peptide or with jasmonic acid, but not in leaves treated with water (Figure 1D).

**The ZmPep1 peptide activates production of jasmonic acid**

To confirm that ZmPep1 acts as a defense regulator, we quantified JA concentrations in excised leaves supplied with water or ZmPep1. After 4 hr ZmPep1 induced the accumulation of JA to levels 4.6-fold higher that of control leaves supplied with water.
To evaluate the dose-dependence of ZmPep1 treatment and subsequent JA accumulation, leaves were treated with increasing concentrations of ZmPep1, ranging from 0.2 to 2000 pMols g\(^{-1}\) fresh weight (FW). After 4 h, JA levels in control leaves supplied with water averaged around half that of leaves supplied with the lowest concentration of ZmPep1 (Figure 2C). Average JA levels increased with the application of increasing amounts of peptide, with a maximum ten times that of water-supplied leaves (Figure 2C). The concentration of ZmPep1 that induced half-maximal JA accumulation fell between 200 fMols and 2 pMols g\(^{-1}\) FW. The vapor phase extraction method followed by GC-MS analysis of JA allowed us to simultaneously measure salicylic acid, levels of which were not observed to change in our experiments.

In excised leaves, expression of the gene encoding allene oxide synthase (AOS) was wound-inducible; however, leaves supplied with ZmPep1 exhibited a 3.8-fold greater induction of AOS transcript than did wounded leaves supplied with water (Figure 2B). Expression of the allene oxide cyclase (AOC) gene was more specifically induced by ZmPep1 treatment. Compared to unwounded leaves at time 0, excised water-supplied leaves displayed modest 5-fold increases in transcript while ZmPep1-treated leaves exhibited a 30-fold induction (Figure 2B). Maximal increases in AOC transcript abundance also occurred at 4 h. Similar to JA production, ZmPep1-induced expression of both the AOS and AOC genes was dose-dependent. At 4 h, relative transcript levels of both genes showed increases in abundance starting at ZmPep1 applications of 20 pMols g\(^{-1}\) FW (Figure 2D).

**ZmPep1 induces ethylene emission**

Given that ET commonly interacts with JA to regulate pathogen defenses, ET production was also investigated. After 2 h, ZmPep1-supplied leaves emitted a 5-fold increase in ET compared to water-supplied leaves (Figure 3A). ZmPep1-induced ET production was dose dependent, and average emissions increased as the amount of peptide supplied to leaves increased (Figure 3B). Expression of the gene encoding 1-aminocyclopropane-1-carboxylic acid oxidase (ACC Ox) also responded to ZmPep1 treatment. ZmPep1 induced an 8-fold increase in transcript levels above those detected in water-treated leaves, which showed no measurable change in ACC Ox expression.
(Figure 3C). Similar to AOS and AOC genes, 20 pMols g$^{-1}$ FW ZmPep1 was observed to be the threshold level for effects on ACC Ox gene expression (Figure 3D). While peak levels of ET emission occurred after 2 h of treatment, increased expression of the ACC Ox gene was greatest after 4 h. This implies that the early induction of ET in ZmPep1-treated leaves occurs either through activation of ACC oxidase enzyme activity or translational activation rather than increases in transcription.

**ZmPep1 regulates expression of pathogen defense genes**

To examine defense processes associated with ZmPep1-activated production of JA and ET, we examined the expression of established defense marker genes (Doehlmann et al., 2008; Erb et al., 2009). Endochitinase A (ECA), pathogenesis related 4 (PR-4), pathogenesis-related maize seed protein (PRms) and peroxidase (PEX) genes have been shown to be pathogen-inducible in microarray experiments (Doehlmann et al., 2008); whereas SerPIN encodes a Bowman-Birk trypsin inhibitor that is strongly induced by JA treatment, elicitors and biotic stresses (Erb et al., 2009).

Expression of all five genes was elevated in excised leaves that had been supplied with ZmPep1. Within 4 h, ECA transcript abundance increased 6-fold in ZmPep1 treated leaves as compared to unwounded control leaves (Figure 4A). After longer treatment times ECA transcripts were also modestly induced by wounding. PEX transcripts demonstrated a 25-fold increase in ZmPep1-treated leaves at 4 h, remaining elevated at 16 h. At 4 h PEX was not strongly wound-inducible, but at later time-points excision resulted in a gradual increase in transcription (Figure 4A). Transcription of PR-4 was wound-responsive in the excised leaves and at early treatment times was induced similarly by both water and ZmPep1 treatment. At 12 h, PR-4 transcripts accumulated to 2-fold higher levels in the ZmPep1-supplied leaves compared to water-supplied controls; however, this response was not statistically significant (Figure 4A). Expression of the gene encoding PRms was modestly but consistently increased 4-fold higher than water-supplied or unwounded control leaves (Figure 4A). At 4 h, ZmPep1 treatment resulted in accumulation of transcript encoding SerPIN, to average levels 50-fold higher than those observed in either excised or unwounded 0 h control leaves (Figure 4A).
For each defense marker gene studied, the induced magnitude of change in transcript abundance was found to be dose-dependent; excised leaves treated with ZmPep1 for 4 h displayed increased defense gene expression with increasing amounts of peptide application (Figure 4B). Changes in transcriptional abundance of the gene encoding PRms were observed at the lowest ZmPep1 treatment level, 200 fMols g⁻¹ FW, while expression of PR-4 was clearly enhanced at 2 pMols g⁻¹ FW. Transcription of the ECA, PEX and SerPIN genes was strongly induced in leaves supplied with 20 pMols g⁻¹ FW ZmPep1.

ZmPep1-induced gene expression was also observed in intact plants using 25 pMols of peptide solution applied to a small wound site (Figure 4C). Transcript abundance of all five defense-associated genes were found to increase in the ZmPep1-treated leaves relative to wounded leaves treated with water, similar to the excised leaf assay. In intact plants, Rhizopus-derived pectinase elicitor also induced increased transcript abundance of each defense gene to comparable levels as ZmPep1 (Figure 4C). Average expression of all five genes was also upregulated in leaf tissue after 24 h of Cochliobolis heterostrophus infection (Figure 4D).

**ZmPep1 promotes accumulation of the defense precursor metabolites anthranilate and indole**

To examine metabolites that fuel the biosynthesis of chemical defenses in maize, we examined benzoxazinoid hydroxamic acid-related precursors (Romero et al., 1995; Frey et al., 2009). ZmPep1 treatment increased the leaf concentrations of both anthranilate and indole after 12 h (Figure 5A). Anthranilate increased from approximately 20 ng g⁻¹ FW in water-treated leaves to more than 700 ng g⁻¹ FW in peptide-treated leaves. Indole increased 30-fold in wounded leaves and more that 1300-fold in ZmPep1-supplied leaves. Accumulation of anthranilate and indole in leaf tissue correlated to the amount of ZmPep1 used to treat the leaves (Figure 5B). Peak induction was achieved at 200 pMols g⁻¹ FW.

To determine whether the increase in anthranilate corresponded with transcriptional regulation of biosynthetic enzymes, expression of the gene encoding anthranilate
synthase subunit 2 (ASsub2) was analyzed. Transcript abundance of ASsub2 increased in ZmPep1-treated leaves relative to water-treated leaves, and was greatest after 4 h of treatment (Figure 5C). Induced expression of the gene was dose-dependent and observable increases in transcript abundance were apparent at peptide concentrations as low as 200 fMol g\(^{-1}\) FW (Figure 5C). Accumulation of both anthranilate and indole occurred in leaves treated with a fungal-derived pectinase elicitor, with observed levels of both metabolites peaking at 12 h (Figure 5D). Infection with \textit{C. heterostrophus} only weakly influenced levels of anthranilate and indole in the leaves at the timepoints examined (Figure 5E).

**ZmPep1 activates biosynthesis of benzoxazinoid defenses.**

Given the above patterns of induced anthranilate, a precursor to indole-derived benzoxazinoid hydroxamic acid defenses, the effect of ZmPep1 on benzoxazinoid metabolism was examined using intact plant assays (Romero \textit{et al.}, 1995; Frey \textit{et al.}, 2009). In both young plants and old plants, there was no significant difference in free 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) or DIMBOA-Glc (Figure 6A). However, 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc), responded modestly to wounding and strongly to peptide treatment.

Young plants had a 3-fold higher basal total hydroxamic acid content than older plants (Figure 6A). Total hydroxamic acid content in young plants was modestly increased by ZmPep1 treatment, but in older plants the total hydroxamic acids more than doubled in response to ZmPep1, indicating that \textit{de novo} hydroxamic acid synthesis was required (Figure 6A). When the ratio of HDMBOA-Glc was compared to DIMBOA and DIMBOA-Glc in leaves, it accounted for an increased percentage of hydroxamic content in both young and old plants, but whereas DIMBOA-Glc predominated in young plants, in old plants HDMBOA-Glc became the predominant hydroxamic acid (Figure 6B).

Production of indole by BX1 is the first committed enzymatic reaction leading into benzoxazinoid synthesis. Expression of the gene encoding BX1 is responsive to biotic stresses, and modulation of \textit{BX1} expression is a mechanism regulating benzoxazinoid pathway activity (Frey \textit{et al.}, 2009; Niemeyer, 2009). To ascertain whether ZmPep1
might activate metabolic flux through the pathway by inducing $BX1$ gene expression. After 4 h of treatment with ZmPep1, $BX1$ transcripts accumulated to 30-fold higher levels than found in time 0 control leaves (Figure 6C). Excised leaves in water also had increased $BX1$ expression, but only 10-fold higher than that of time 0 control leaves.

**ZmPep1 enhances resistance to southern leaf blight disease**

Because ZmPep1 activates production of JA, ET, expression of pathogen defense genes and accumulation of HDMBOA-Glc, we hypothesized that pretreatment of plants would improve plant disease resistance. To test this hypothesis, intact plants were treated with water or with ZmPep1 18 h prior to inoculation with *Cochliobolis heterostrophus*, a fungal necrotroph that is the causative agent of southern leaf blight. Chlorotic lesions spread from the wound sites of infected leaves that had been pretreated only with water after 3 d (Figure 7A). In the ZmPep1-pretreated leaves, lesions were contained at the edge of the wound site and had not spread. *C. heterostrophus*-induced lesion area in ZmPep1-treated leaves was less than half that of water-treated leaves even at high inoculation loads of *C. heterostrophus* (Figure 7B).

Leaves that had been pretreated with water had increased cell death, as estimated by ion leakage, relative to leaves that had been pretreated with ZmPep1 (Figure 7C). As spore inoculation levels increased, subsequent average ion leakage from the infected leaves also increased. Across all inoculum levels, ZmPep1 pretreated leaves were more resistant to *C. heterostrophus*-induced cell death. At the lowest concentration of fungal inoculum applied, percent ion leakage was 20-fold less in ZmPep1-pretreated leaves as compared to water controls.

**ZmPep1 enhances resistance to anthracnose stalk rot**

To examine ZmPep1 activity in stems, resistance to anthracnose stalk rot was examined. In plants pretreated with water, the progression of lesion spread from the infected node was less than in control plants, indicating that wounding alone enhanced resistance (Figure 8A). As compared to either untreated or water-treated plants, the nodes of ZmPep1-treated plants displayed very little necrotic spread.
After 4 d greater than 90% of the stalk area was necrotic in untreated control stems that had been inoculated with $3.3 \times 10^3$ C. graminicola conidia (Figure 8B). Stalks of plants that were pretreated with water were 45-50% necrotic at high inoculation levels whereas ZmPep1-pretreatment resulted in only 25% stem rot. Measurements of conductivity to ascertain the extent of cell death as indicated by ion leakage revealed a similar trend. ZmPep1-pretreated stalks had less than a 100 µS cm$^{-2}$ increase, less than half that of water-pretreated stems and a quarter that of directly infected controls (Figure 8C).

**ZmPep1 is an active signal in several varieties of maize**

To elucidate whether ZmPep1 modulates responses in other Z. mays varieties, excised leaves of pathogen-resistant lines HI-27 and MP313E were supplied with peptide and levels of JA and indole were quantified. JA accumulated in leaves of all three maize lines after 4 h of ZmPep1 treatment (Figure 9A). Similarly, indole was observed to increase in ZmPep1-treated leaves of the three lines (Figure 9B). For both MP313E and HI-27, the magnitude of indole production was less than in Golden Queen, but the peptide-treated leaves were observably induced compared to water-treated leaves. It may be that these varieties are less sensitive to ZmPep1 as a signal, or that the selection of indole as a defense marker metabolite is not ideal for all maize lines.

**Discussion**

We demonstrate that ZmPep1 acts as a defense-regulating signal and extend characterization of this family of peptides beyond Arabidopsis. This work examined the molecular and biochemical defenses induced by ZmPep1 that are collectively associated with resistance against invading microorganisms. The maize ZmPROPEP1 ortholog of AtPROPEP1 is functionally homologous, and the gene is transcribed in response to both JA and pathogen infection. As does AtPep1, the ZmPep1 peptide activates numerous components of the innate immune response. This maize peptide-activated defense response was characterized by production of defense-related phytohormones, induced expression of pathogen defense genes, accumulation of benzoxazinoid defenses and enhanced resistance to multiple pathogens.
Like other endogenous peptide regulators of defense, ZmPep1 functions through activation of oxylipin signaling, inducing both expression of JA biosynthetic genes and JA accumulation (Howe et al., 1996; Huffaker & Ryan, 2007). Furthermore, ET is also a component of ZmPep1-signaling; the peptide activates expression of the gene encoding ACC oxidase and promotes ET emission in a dose-dependent manner. Coordinated activity of JA and ET signaling regulates pathogen defense responses in many plants (Bari and Jones, 2009; Glazebrook, 2005; Rojo et al., 2003). While the molecular mechanisms regulating pathogen defense responses in maize are not as well characterized as plants such as Arabidopsis, evidence is accumulating that indicates cooperative JA/ET signaling is a conserved motif of defense initiation. Both JA and ET are produced by maize in response to biotic stress and insect elicitor treatment (Schmelz et al., 2003; Schmelz et al., 2009). Additionally, elicitor-modulated JA/ET signaling by Trichoderma virens is proposed as the mechanism by which this beneficial fungus activates induced systemic resistance in maize (Djonovic et al., 2007). Our results demonstrating that ZmPep1 regulates JA, ET and pathogen resistance supports the cooperative role of these hormones as signals for maize pathogen defense.

In addition to mediating production of JA and ET, ZmPep1 also promoted increased transcript abundance for genes encoding antimicrobial and defense signaling proteins. Consistent with the proposed role of ZmPep1 as an endogenous elicitor, many defense-related transcripts were also induced by both infection with C. heterostrophus the exogenous fungal elicitor pectinase. The PR-4 and ECA genes regulated by ZmPep1 are activated by pathogen attack and encode chitinase proteins likely to have direct antifungal activity through degradation of fungal cell walls. In germinating maize embryos, PR-4 gene expression is stimulated by inoculation with fungi and by fungal elicitor extracts; and it is inducible in leaves by JA, abscisic acid and wounding (Bravo et al., 2003). Both PR-4 and ECA transcripts also accumulate in Ustilago maydis-infected ears (Doehlemann et al., 2008, Bravo et al., 2003).

In addition to genes encoding antimicrobial PR proteins, ZmPep1 induced expression of the PRms gene, a homolog of the tobacco PR-1 family that is induced by fungal infection (Casacuberta et al., 1992). Rather than having direct antimicrobial activity,
PRms acts as a defense regulator. In both rice and tobacco, constitutive PRms gene expression was found to increase basal levels of defense gene transcripts and to confer enhanced resistance to infection by several pathogens (Murillo et al., 2003; Gómez-Ariza et al., 2007). This upregulation of defense by PRms is proposed to occur through modulation of sucrose-mediated signaling, raising the intriguing possibility that in addition to activating defense through JA/ET hormone signaling, ZmPep1 may promote disease resistance through PRms-mediated sugar signaling events as well (Gómez-Ariza et al., 2007).

ZmPep1-induced PEX may detoxify reactive oxygen species generated through cellular damage or signaling, or crosslink lignin, cellulose and extensin to strengthen cell walls against attacking organisms (Lagrimini et al., 1987; Hiraga et al., 2001). SerPIN may act in direct defense since it is a serine proteinase inhibitor that could inhibit digestive proteases from both insect and microbial invaders (Ryan, 1989). However, serpin family proteins are also regulators of proteolytic signaling cascades required for innate immune responses in mammals and insects (Law et al., 2006). Furthermore, a serpin in Drosophila, termed Necrotic, modulates signaling by spätzle, an endogenous peptide signal mediating Drosophila innate immune responses (Levashina et al., 1999). It remains to be determined whether ZmPep1-induced SerPIN acts in direct defense or as a signaling modulator.

While the antimicrobial and signaling-related genes upregulated by ZmPep1 are likely factors contributing to induced disease resistance, small molecule defenses are also likely to contribute. Benzoxazinoids are indole-derived hydroxamic acid defenses in Poaceous plants that are associated with herbivore and pathogen resistance (Niemeyer, 2009). Cellular damage caused by attacking organisms releases reactive benzoxazinoids from their glycosylated precursors (Frey et al., 2009). Maize seedlings and young tissues have relatively high concentrations of DIMBOA and the glucoside DIMBOA-Glc which are believed to help protect these essential tissues; however, the role of benzoxazinoids in older plants is not as well-defined (Neimeyer, 2009).

Neither DIMBOA-Glc nor free DIMBOA were found to accumulate in response to ZmPep1, but HDMBOA-Glc was induced in ZmPep1-treated leaves. The second
methoxyl group on HDMBOA renders the molecule less stable and more reactive than DIMBOA (Maresh et al., 2006). With respect to invading organisms, HDMBOA seems to have multiple functions, capable of acting as both a toxin and a negative effector of pathogenicity. HDMBOA-Glc is a component of maize defense against southwestern corn borer (SWCB), *Diatraea grandiosella*, in resistant varieties (Hedin et al., 1993). SWBC-resistant maize lines are enriched in HDMBOA content compared to susceptible lines and HDMBOA was shown to be directly toxic to larvae. HDMBOA is also a predominant constituent of maize root exudates and is postulated to generate a continuously maintained defensive zone in the soil surrounding the roots (Zhang et al., 2000). In root studies, HDMBOA did not act to prevent colonization of roots by *Agrobacterium tumefaciens*, but was found to decompose into an o-imidoquinone intermediate that inhibited *A. tumefaciens* virulence gene expression (Maresh et al., 2006).

Specific accumulation of HDMBOA-Glc is inducible in both wheat and maize by treatment with JA, pathogen infection and herbivory (Bücker and Grambow 1990; Oikawa et al., 2001; Oikawa et al., 2002; Oikawa et al., 2004). In these studies, accumulation of HDMBOA-Glc seemed to occur in direct correlation to reduced levels of DIMBOA-Glc, implying that HDMBOA-Glc was generated through methoxylation of existing DIMBOA-Glc pools rather than through *de novo* hydroxamic acid biosynthesis (Oikawa et al., 2001). For ZmPep1-induced HDMBOA-Glc accumulation, the proportion of HDMBOA-Glc relative to DIMBOA-Glc was increased; however, the increase in HDMBOA-Glc did not come at the expense of DIMBOA-Glc. Rather, we observed that total hydroxamic acid content increased in the ZmPep1-treated leaves of older plants, indicating that the peptide activated *de novo* synthesis of benzoxazinoids that was channeled into HDMBOA-Glc production. ZmPep1-induced expression of the *BX1* gene, encoding an indole glycerol lyase that catalyzes the first committed step in benzoxazinoid production, also supports enhanced metabolic flux into the pathway (Frey et al., 2009; Melanson et al., 1997).

Manipulation of innate immune responses by ZmPep1 caused enhanced disease resistance. ZmPep1-treated plants displayed decreases in both lesion size and cell
death in leaves challenged with the necrotroph *C. heterostrophus* and in stems challenged with the hemibiotroph *C. graminicola*. Mechanisms of maize resistance to both of these pathogens are still poorly understood. *C. heterostrophus* is divided into two subgroups based upon toxin production, race T which produces toxin, and race O which does not. Race O is an endemic pathogen in hot and humid climates and continues to cause disease resulting in lost yield, particularly along the south Atlantic coast (Byrnes et al., 1989). Although a single recessive locus, rhm1, exists that can confer resistance to SLB through an unknown mechanism, most maize lines currently grown rely upon additive quantitative traits that confer partial resistance (Simmons et al., 2001; Balint-Kurti and Carson, 2006). Similar to *C. heterostrophus*, resistance to *C. graminicola* is also primarily quantitative and polygenic (Venard and Vaillancourt, 2007). *C. graminicola* is common in maize fields across the US and while the fungus is able to infect most maize tissue, it primarily causes yield losses due to anthracnose stalk rot (Bergstrom and Nicholson, 1999; Venard and Vaillancourt, 2007).

*Colletotrichum* species are known to actively evade and suppress plant defense, but the fungus was unable to overcome the defense responses pre-activated by ZmPep1 treatment (Münch et al., 2008). Transgenic Arabidopsis plants constitutively expressing *AtPROPEP1* exhibited increased basal levels of the same genes that were induced in wild type plants by treatment with AtPep1 (Huffaker et al., 2006). This constitutive induction of basal immunity resulted in increased pathogen resistance (Huffaker et al., 2006). Transgenic maize plants constitutively expressing the *ZmPROPEP1* gene may also display higher basal levels of the genes and metabolites that were observed in plants ectopically treated with ZmPep1 peptide. Several molecular studies of maize resistance to attacking organisms have indicated that resistance is associated with increased basal levels of defense gene expression and defense metabolite accumulation similar to those induced by ZmPep1 (Allesandra et al., 2010; Niemeyer, 2009; Hedin et al., 1993). The ability of ZmPep1 to elicit defense signaling and metabolite accumulation in multiple maize lines indicates constitutive expression through transgenic means could yield results across varieties.
Because endogenous peptide regulators such as ZmPep1 activate multiple defense pathways rather than one gene or metabolite, they may provide a potentially useful strategy to contribute to quantitative resistance through manipulation of a single gene. In crop plants, quantitative disease resistance relies on the additive effects of multiple defenses to provide broad spectrum partial resistance to many different pathogens (Wisser et al., 2006; Poland 2009). Although quantitative resistance is highly desirable, direct incorporation of this trait into crop development is difficult because of its combinatorial nature; the additive effects that make this resistance robust and versatile also make it difficult to manipulate. Transgenic modulation of peptide signaling has already shown promise as a mechanism for manipulating quantitative resistance. For example, the gene encoding EFR, a Brassicaceous-specific pattern recognition receptor that binds a bacterial peptide MAMP to elicit broad innate immune responses, was ectopically expressed in *Nicotiana benthamiana* and tomato (Lacombe et al., 2010). Transgenic expression of this receptor enhanced resistance to diverse bacterial species by facilitating recognition of attack and activation of a broad spectrum of defense responses. Constitutive expression of the *ZmPROPEP1* gene could similarly confer quantitative resistance effects through simultaneous upregulation of basal levels of defense responses in maize plants. Furthermore, because orthologs of the precursors to AtPep1 and ZmPep1 have been identified across the plant kingdom, this strategy of endogenous-peptide manipulation of defense responses could potentially be used to enhance disease resistance in many diverse plant species.

**Materials and methods**

**Plant and fungal materials**

*Zea mays* varieties used were B73, HI-27, MP313E and Golden Queen. All were potted in professional grower’s soil mix (Piedmont Pacific, Statham, GA) blended with 14–14-14 Osmocote (Scotts, Marysville, OH). All varieties were cultivated in a greenhouse under the following conditions: 12 hour photoperiod, with a minimum of 300 µmol m⁻² s⁻¹ of photosynthetically active radiation supplied by supplemental lighting. Relative humidity was maintained at 70% and temperature cycled between 24°C at night and 28°C during the day.
*Cochliobolus heterostrophus* was isolated from leaf material of an infected maize plant growing in Gainesville, Fl. The specimen was streaked on 1/2x potato dextrose agar (PDA) and subcultured until pure isolates were obtained. The fungus was identified by the Florida Extension Plant Disease Clinic at the University of Florida through macroscopic colony appearance, examination of morphology under both dissecting and light microscopes, and by PCR analysis of fungal DNA with species-specific primers. Spore suspensions of *C. heterostrophus* were prepared in 30% glycerol/0.1% TWEEN and stored at -80°C. For each bioassay, an aliquot of glycerol stock was used to generate a fresh working culture on 1/2x PDA (Sigma-Aldrich, St. Louis, MO) that was incubated for two weeks at 26 ºC. *Colletotrichum graminicola*, strain M1.001 was acquired from Dr. Jeffrey Rollins (Dept. of Plant Pathology, University of Florida) and conidial spore stocks were prepared in 30% glycerol/0.1% TWEEN and stored at -80°C. For each assay, a fresh working culture was prepared by spotting glycerol stock onto V8 Agar plates and incubated for 1.5 to 2 weeks at 26 ºC.

**Peptide and precursor gene identification**

The previously identified AtPROPEP1 sequence (Huffaker *et al.*, 2006) was used to query GenBank registered nucleotide sequences from *Zea mays* through the National Center for Biotechnology Information (NCBI) TBLASTN version 2.2.7 algorithm (Altschul *et al.* 1997). Alignments with the AtproPep1 sequence revealed a Gen Bank accession, DY240150, a full length cDNA that encodes the ZmPROPEP1 precursor in the -1 frame. To determine possible localization of the protein in the cell the pSORT prediction program was used (Nakai and Kanehisa 1991).

**Peptide synthesis**

A 23 amino acid peptide corresponding to the predicted ZmPep1 active peptide sequence, VRRRPTTPGRREGSGGNGNH, was synthesized by solid-phase peptide synthesis at the Protein Core Chemistry Facility, University of Florida, Gainesville, using *N*- (9-fluorenlymethoxycarbonyl)-protected amino acids on a 432A Peptide Synthesizer (Applied Biosystems, Carlsbad CA). The peptide was cleaved from the resin with modified reagent K, and HPLC-purified on a RP-C18 column using an
water:acetonitrile gradient in 0.1% trifluoroacetic acid. The peptide was confirmed to be of the expected molecular weight (2452.63) by mass spectrometry.

Nucleic acid purification and isolation

DNA was isolated from *Z. mays* leaves using the genomic DNA isolation reagent DNAzol (Invitrogen, Carlsbad CA) as per the instructions provided with the reagent. For RNA isolation, tissues that had been harvested and frozen in liquid nitrogen were ground to a fine powder and approximately 100 mg of frozen powdered plant material was extracted in 1 mL of Trizol reagent (Invitrogen, Carlsbad CA). RNA isolation was performed as per the Trizol instructions, supplemented by an acid:phenol:chloroform partitioning step to minimize contaminating DNA.

Cloning of the Zm\textit{PROPEP1} gene and cDNA

RNA isolated from young maize leaves was reverse transcribed using the RETROscript kit (Applied Biosystems, Carlsbad CA) as per kit instructions with random decamer primers. The Zm\textit{PROPEP1} open reading frame was amplified from the cDNA with the forward primer 5’-GACCTCAGGAAAGGGGAGACCTGGA-3,’ and reverse primer 5’-AAGGAAGCGAACAAGCTAGGGTCACCGTA-3’ using Phusion Hot Start II DNA Polymerase (New England Biolabs, Ipswich, MA). The amplified cDNA was cloned into the pCR BLUNT II TOPO vector using a Zero Blunt PCR cloning kit (Invitrogen, Carlsbad CA) as per kit instructions and transformed by heat shock into TOP10F’ chemically competent *E. coli* (Invitrogen, Carlsbad CA). Colonies were screened by PCR using the Zm\textit{PROPEP1} primers and plasmids from positive colonies were sequenced using ABI Prism BigDye terminator (Applied Biosystems, Foster City CA). All sequencing reactions were run at the DNA Sequencing Core Facility (University of Florida, Gainesville, FL).

Leaf bioassays for analysis of transcript and metabolite abundance

For excision assays, leaf five of three week old maize plants was cut and placed in four mL glass vials containing either water, or a ZmPep1 solution in water. For each treatment and time point six leaves of leaf stage five were assayed. At the time points
indicated, the entire leaf was harvested in liquid nitrogen for RNA and metabolite analysis. Zero hour control leaves were harvested directly from the plant into liquid nitrogen. For intact leaf assays, wax was gently scraped from leaves at two sites on either side of the midrib on leaf five of three week old plants. Five µL of water or of solutions in water of 25 pMol ZmPep1, 500 µg pectinase elicitor or 5 x 10^3 fungal spores was applied to each site. After the time indicated, a 7.5 cm segment of leaf surrounding the wound sites was harvested in liquid nitrogen for RNA and metabolite analysis.

**Semi-quantitative RT-PCR**

RNA was reverse transcribed using RETROscript reagents (Applied Biosystems, Carlsbad CA) with reactions assembled and incubated as per kit instructions. Semi-quantitative PCR was assembled as follows: Template cDNA was used at 120 ng per reaction. Each 25 µL reaction had 0.5 units Platinum Taq polymerase diluted into Platinum 10x PCR buffer (Invitrogen, Carlsbad CA) with 1.5 mM Mg2+, 200 µM each dNTP and 0.4 µM each primer. All primers were designed to be used at an annealing temperature of 56ºC, amplify regions 150 to 350 base pairs in length and to span introns when possible; primer sequences are listed in Table 1. The Actin1 gene transcript (GenBank# J01238) was used to permit comparisons of relative transcript abundance from sample to sample (Kirchberger et al., 2007; Erb et al., 2009). PCR was performed as follows: 3 min at 94ºC, 30 s at 94ºC, 30 s at 56ºC, 1 min at 72ºC; final 10 min at 72ºC. Cycling time for each transcript was optimized and ranged between 25 and 38 cycles. The number of amplification cycles used for each is listed in Supplementary Table 1.

A 20 µL aliquot of each reaction product was diluted with 2 µL DNA blue/orange loading dye (Promega Biosciences Inc., San Luis Obispo CA) and analyzed electrophoretically on a 1% agarose/Tris-Acetate-EDTA gel impregnated with ethidium bromide (Promega Biosciences Inc., San Luis Obispo CA). The gel was visualized on a Gel Doc XR Imaging System (BioRad, Hercules, CA) using Quantity One version 4.6.2 software (BioRad, Hercules, CA). A high resolution image of the gel was captured and band intensity measured using the Quantity One program (BioRad, Hercules, CA). Band intensity of each transcript was normalized by dividing the measured value by the value
obtained from measurement of actin band intensity for the same sample. Values obtained for estimation of relative transcript abundance were then defined as fold change in normalized band intensity for each treatment versus normalized band intensity of an untreated control sample.

**Measurement of hormones and metabolites**

Levels of JA, indole and anthranilic acid were measured using the previously described vapor phase extraction method with GC-MS analysis (Schmelz *et al.*, 2004). Quantification of indole levels in each sample were performed by comparison to an external standard curve. Ethylene emitted by leaves was measured by GC as previously described (Schmelz *et al.*, 2009).

**Analysis of benzoxazinoid phytoalexins**

Benzoxazinoids were extracted and analyzed by HPLC as described by Erb *et al.* (2009). After 24-h, leaf tissue surrounding the treatment sites was harvested in liquid nitrogen, freeze-dried and extracted in 49:1 methanol:acetic acid prior to analysis. Quantities were estimated using 6-hydroxy-2(3H)-benzoxazolone as an internal standard. HDMBOA-glc is the predominant hydroxamic acid in 20-d maize old roots (Cambier *et al.*, 2000); thus, root tissue was used to confirm the HPLC retention time of this ZmPep1-induced metabolite in leaves. HDMBOA is known to be highly unstable (Maresh *et al.* 2006). Unlike DIMBOA-glc, even low levels of water in the extracted tissue caused the complete loss of analyzable HDMBOA-glc.

**Leaf blight resistance assays**

Intact 2.5 week-old maize plants were infected with *C. heterostrophus* as follows: On leaves five and six, the wax was gently scraped from each leaf twice on both sides of the midrib and five µL of water or 25 pMol ZmPep1 was applied to each wound site and allowed to air-dry. After 12 to 24 hours, five µL of *C. heterostrophus* spores in a 0.1% TWEEN 20 solution was applied to each wound site and let dry. Each plant was then incubated in open-bottomed glass chambers under greenhouse lights for 3 days with 100% humidified air passed over each plant at 4 L min⁻¹. After 3 days, leaves were
photographed and the lesion area measured using ASSESS 2.0 Image analysis software for Plant Disease Quantification by Lakhdar Lamari (American Phytopathological Society). The extent of cell death was estimated through measurement of ion leakage as described by Torres et al. (2002). Briefly, 4 leaf disk samples, each with an area of 1 cm², were collected from infected or uninfected tissues, immersed in 4 mL of water and vacuum infiltrated for 1 min. After shaking gently for 1 h, the conductivity of the samples was measured in µSiemens at 25 °C using a YSI 3100 conductivity meter (YSI Incorporated, Yellow Springs, OH). To measure total potential conductivity of each sample, the leaf discs in water were microwaved for 1 min, and after cooling to 25 °C, the conductivity was remeasured. A comparison of initial conductivity to total potential conductivity of the same leaf discs resulted in a number expressed as a percent of total conductivity for each sample. *C. heterostrophus*-induced ion leakage was then defined as the difference between percentage of total conductivity measured for *C. heterostrophus*-infected samples and that of samples from uninfected leaf tissue in the same assay.

**Stalk rot resistance assays**

A 1.0 mm diameter cork-borer was used to bore a hole through the second above-ground node in the stalk of 3.5 to 4 week old plants. The hole was filled with 10 μL of either water or 50 pMol ZmPep1 to eliminate any air bubbles. A plastic pipette-tip filled with 1 mL of either water or 5 nMol ZmPep1 was then gently inserted into the hole until it was secure. The plant was allowed to take up the full 1 mL of pretreatment solution, which typically occurred within 2 h. After a 3 h pretreatment, the pipette tips were removed and the hole was inoculated with 10 μL of a *C. graminicola* spore suspension in sterile water. For untreated control plants a hole was bored through the second node at the time of inoculation. After 4 d stems were split open, photographed and lesion area determined using ASSESS 2.0 Image analysis software. A 12.5 cm segment centered around the inoculated node was cut, immersed in 15 mL of water, vacuum-infiltrated for 1 min, and after 1 h of incubation, ion leakage was measured as described by Torres et al. to estimate the extent of cell death (Torres et al., 2002). Values obtained for *C. graminicola*-induced ion leakage were defined as the ratio of
conductivity values measured in infected stem samples compared to conductivity measured in wounded control stem samples.

**Statistical Analysis**

Analyses of variance (ANOVAs) were performed on the quantified levels of metabolites, transcripts, pathogen lesion size and ion leakage estimates. Treatment effects were investigated when the main effects of the ANOVAs were significant ($P < 0.05$). Where appropriate, Tukey tests were used to correct for multiple comparisons between control and treatment groups. T-tests were also used in limited specific cases to examine significant differences in treatments groups compared with selected controls. With the exception of percentage data, prior to statistical analysis, all data was subjected to square root transformation to compensate for elevated variation associated with larger mean values. The analysis was accomplished with JMP 4.0 statistical discovery software (SAS Institute, Cary, NC).

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ASSESS 2.0 Image analysis software for Plant Disease Quantification by Lakhdar Lamari © 2008 American Phytopathological Society


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Figure Legends

**Figure 1.** Comparison of the proteins encoded by the AtPROPEP1 and ZmPROPEP1 genes. A, Conserved precursor motifs are EKE motif (blue), amphipathic helix motif (purple), bioactive elicitor peptide (red, underlined); B, Comparison of conserved characteristics within the active AtPep1 and ZmPep1 peptides. Basic residues are blue, identical amino acids are red. C, Average \((n = 3, \pm \text{SEM})\) induced ZmPROPEP1 gene expression in leaves by the fungal pathogen C. heterostrophus. D, Average \((n = 3, +\text{SEM})\) induced expression of the ZmPROPEP1 precursor gene in response to treatment of intact leaves with ZmPep1 or JA. In C, different letters (a-d) represent significant differences within the plot. In D, different letters (a,b) represent significant differences within each time point. Different letters (a-d) represent significant differences within the plot. (all ANOVA \(Ps < 0.005\); Tukey test corrections for multiple comparisons; \(P < 0.05\)).

**Figure 2.** ZmPep1 induces JA accumulation and regulates expression of related biosynthetic genes. Time-course analysis of A, JA levels and B, AOS & AOC gene expression in excised leaves supplied with water or ZmPep1 (2 nMols g\(^{-1}\) FW). Relative transcript abundance levels were examined using semi-quantitative PCR with actin as a control; C, Dose dependence of JA levels and D, AOS & AOC gene expression in response to ZmPep1 at 4 h. Each sample was a pool of two leaves \((n = 3, \pm \text{SEM})\). At the time point of greatest mean change, different letters (a-b) represent significant differences (all ANOVA \(Ps < 0.02\); Tukey test corrections for multiple comparisons where applicable; \(P < 0.05\)).

**Figure 3.** ZmPep1 regulates emission of ET and expression of an ET biosynthesis gene. A, Time course of average \((n = 4; \pm \text{SEM})\) induced ET emission in excised leaves supplied with water or with ZmPep1 (2 nMol g\(^{-1}\) FW). B, Dose-dependence of average \((n = 4; +\text{SEM})\) ZmPep1-triggered ET emission at 2 h; C, Time course of average \((n = 3; \pm \text{SEM})\) ACC oxidase transcriptional changes in response to water or ZmPep1 (2 nMol g\(^{-1}\) FW); D. Concentration effects of ZmPep1 on induced ACC oxidase expression at 4 h. Relative transcript abundance levels were examined using semi-quantitative PCR with actin as a control. Different letters (a,b) represent significant differences (all ANOVA \(Ps < 0.05\), Tukey test corrections for multiple comparisons where applicable; \(P < 0.05\)).

**Figure 4:** ZmPep1 activates defense gene expression. A, Average fold changes in transcript abundance over time in excised leaves treated with water or ZmPep1 (2 nMol g\(^{-1}\) FW) versus untreated leaves; B, Dose-dependence of ZmPep1-induced transcriptional changes in excised leaves at 4 h with actin amplification as a control; C, Local average changes in defense gene transcription in intact plants 4 h after application of water, 25 pMols ZmPep1 or pectinase elicitor to a wound site; D. Local average changes in transcript abundance in intact plants 24 hours after inoculation with \(5 \times 10^{3}\) C. heterostrophus spores. For graphs in A, at the time point of greatest mean change different letters (a, b) represent significant differences with ANOVA P values <
0.02 (Tukey test corrections for multiple comparisons; \( P < 0.05 \)). For graphs in C and D, stars (*) represent significant differences from water-treated control (\( P < 0.05 \)). Not statistically different (nsd) indicates ANOVA P values > 0.05. For all graphs, \( n = 3, \pm \) SEM.

**Figure 5.** ZmPep1-induced defense-associated metabolites. A, Average anthranilate and indole accumulation in excised leaves B, Dose-dependence of ZmPep1-induced anthranilate and indole accumulation in excised leaves; C, Time course and dose-dependence of ZmPep1-induced changes in expression of the ASsub2 gene. Transcript abundance was normalized via comparison to an actin control and expressed as fold change relative to an untreated leaf. For all experiments, unless otherwise indicated, ZmPep1 was supplied at 2 nMol g\(^{-1}\) FW. Each sample was a pool of two leaves, \( n = 3 \), error bars represent SEM. For graphs in A and C, at the time point of greatest mean change different letters (a, b) represent significant differences with ANOVA P values < 0.02 (Tukey test corrections for multiple comparisons; \( P < 0.05 \)). For graphs in B, stars (*) represent significant differences from water-treated control (\( P < 0.04 \)). For all graphs, \( n = 3, \pm \) SEM.

**Figure 6.** Effect of ZmPep1 treatment on benzoxazinoid defenses. A, Levels of benzoxazinoids in intact leaves after 24 h treatment with water or 25 pMols ZmPep1; \( n = 4, \pm \) SEM. B, Relative ratios of benzoxazinoids to one another in untreated plants versus water and ZmPep1-treated; C. Timecourse of transcript accumulation of BX1 in leaves treated with water or ZmPep1. Relative transcript abundance was measured by semiquantitative RT-PCR with normalization to an actin control; \( n = 3, \pm \) SEM. For all graphs in A, different letters (a, b) represent significant differences with ANOVA P values < 0.02 (Tukey test corrections for multiple comparisons; \( P < 0.05 \)). Not statistically different (nsd) indicates ANOVA P values > 0.05. For the graph in C at the time point of greatest mean change different letters (a, b) represent significant differences with ANOVA P values < 0.05 (Tukey test corrections for multiple comparisons; \( P < 0.05 \)). Not statistically different (nsd) indicates ANOVA P values > 0.05. For all graphs, \( n = 3, \pm \) SEM.

**Figure 7.** ZmPep1 pretreatment induces resistance to *C. heterostrophus* (southern leaf blight disease, SLB). A, Lesions in leaves pretreated with water or with 25 pMols of ZmPep1 three days after SLB infection; B, Average lesion area in plants pretreated with water or ZmPep1; C, Average SLB-induced cell death in samples from water or ZmPep1 pretreated leaves as measured by the average percent increase in ion leakage versus samples from uninfected leaves. For lesion analysis \( n = 16, \pm \) SEM; for ion leakage, samples were pools of 4 leaves; \( n = 4, \pm \) SEM. For both graphs, different letters (a-e and a-c) represent significant differences with ANOVA P values < 0.001 (Tukey test corrections for multiple comparisons; \( P < 0.05 \)).

**Figure 8.** ZmPep1-induced resistance to anthracnose stalk rot caused by *C. graminicola*; A, Necrosis in stems that were pretreated for 3 h with water or 5 nMol ZmPep1; B, Percent of stem rotted 4 d after infection; C, *C. graminicola*-induced cell death as measured by ion leakage. For data shown, \( n = 5 \) with error bars representing
SEM. For graph in B, stars (*) represent significant differences from water-treated control (P < 0.002), at the time point of greatest mean change. For graph in C, different letters (a-c) represent significant differences with ANOVA P values < 0.001 (Tukey test corrections for multiple comparisons; P < 0.05).

**Figure 9.** ZmPep1 promotes production of jasmonic acid (JA) and defense-related metabolites in multiple maize varieties. A, Time course of induced JA in excised leaves supplied with water or ZmPep1; B, Indole measured in excised leaves; C, Anthranilate levels in excised leaves. ZmPep1 was supplied at 2 nMol g\(^{-1}\) FW. Samples were pools of two leaves, n = 3; error bars are SEM. For graphs in A, different letters (a-c) represent significant differences with ANOVA P values < 0.001 (Tukey test corrections for multiple comparisons; P < 0.05). For graphs in B, stars (*) represent significant differences from water-treated control (P < 0.05).
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Figure 4: ZmPep1 activates defense gene expression. A, Average fold changes in transcript abundance over time in excised leaves treated with water or ZmPep1 (2 nMol g⁻¹ FW) versus untreated leaves; B, Dose-dependence of ZmPep1-induced transcriptional changes in excised leaves at 4 h with actin amplification as a control; C, Local average changes in defense gene transcription in intact plants 4 h after application of water, 25 pMols ZmPep1 or pectinase elicitor to a wound site; D, Local average changes in transcript abundance in intact plants 24 h after inoculation with 5 x 10³ C. heterostrophus spores. In A, at the time point of greatest mean change, different letters (a,b) represent significant differences (all ANOVA Ps < 0.02). In C and D, asterisks (*) denote significant differences from water-treated controls (all ANOVA and t-test (LSD) Ps < 0.05). Not statistically different (n.s.d.) indicates ANOVA Ps > 0.05. For all graphs; n = 3, ±SEM.
Figure 5. ZmPep1-induced defense-associated metabolites. A, Average anthranilate and indole accumulation in excised leaves; B, Dose-dependence of ZmPep1-induced anthranilate and indole accumulation in excised leaves; C, Time course and dose-dependence of ZmPep1-induced changes in expression of the ASub2 gene. Transcript abundance was normalized via comparison to an actin control and expressed as fold change relative to an untreated leaf. Unless otherwise indicated, ZmPep1 was supplied at 2 nMol g\(^{-1}\) FW. For all graphs, \(n = 3\), ± SEM. In A and C, at the time point of greatest mean change, different letters (a,b) represent significant differences (all ANOVA \(P_s < 0.02\)). In B and D, asterisks (*) denote significant differences from water-treated controls (all ANOVA and t-test (LSD) \(P_s < 0.05\)). Not statistically different (n.s.d.) indicates ANOVA \(P_s > 0.05\).
Figure 6. Effect of ZmPep1 treatment on benzoxazinoid defenses. A, Average (\(n=4\), ±SEM) benzoxazinoids including DIMBOA-Glc, DIMBOA, and HDMBOA-Glc in intact leaves after 24 h treatment with water or 25 pMols ZmPep1; B, Relative ratios of benzoxazinoids to one another in untreated plants versus water and ZmPep1-treated; C. Time course of average (\(n=3\), ±SEM) BX1 transcript accumulation in leaves treated with water or ZmPep1. Relative transcript abundance was measured by semiquantitative RT-PCR with normalization to an actin control. In A, different letters (a, b) represent significant differences within each separate hydroxamic acid (Hx) analyzed (all ANOVA Ps < 0.04; Tukey test corrections for multiple comparisons; \(P<0.05\)). Not statistically different (n.s.d.) indicates ANOVA \(P\) values > 0.05. In C, at the time point of greatest mean change, different letters (a, b) represent significant differences (ANOVA \(P < 0.05\)).
Figure 7. ZmPep1 pretreatment induces resistance to *C. heterostrophus*. A, Lesions in leaves pretreated with water or with 25 pMols of ZmPep1 3-d after *C. heterostrophus* infection; B, Average (*n* = 16, ±SEM) lesion size in plants pretreated with water or ZmPep1; C, Average induced cell death in samples from water or ZmPep1 pretreated leaves as measured by the average percent increase in ion leakage compared to samples from uninfected leaves. For ion leakage, each sample was a pool from 4 individual leaves (*n* = 4, ±SEM). Within each plot, different letters (a-e) represent significant differences (all ANOVA *P*s < 0.001, Tukey test corrections for multiple comparisons; *P* < 0.05).
Figure 8. ZmPep1-induced resistance to anthracnose stalk rot caused by *C. graminicola*; A, Necrosis in stems that were pretreated for 3 h with water or 5 nMol ZmPep1; B, Average (*n* = 5; ±SEM) percent of stem rotted 4 d after infection; C, Average (*n* = 5; +SEM) *C. graminicola*-induced cell death as measured by ion leakage. In B, asterisks (*) denote significant disease symptom inhibition compared to water-treated controls at selected spore concentrations (ANOVA *P* < 0.001 and t-test (LSD) *P* ≤ 0.05). In C, different letters (a-c) represent significant differences (ANOVA *P* < 0.001; Tukey test correction for multiple comparisons; *P* < 0.05).
Figure 9. ZmPep1 promotes production of JA and a defense-related metabolite in multiple maize varieties. Average (n = 3; ±SEM) A, JA and B, indole in excised leaves treated with either water alone or ZmPep1 supplied at 2 nMol g⁻¹ FW. In A, different letters (a-c) represent significant differences (all ANOVA Ps < 0.001, Tukey test corrections for multiple comparisons; P < 0.05). In B, asterisks (*) denote significant differences within time points from water-treated controls (all ANOVA Ps < 0.02 and t-test (LSD) Ps < 0.05).