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USING A DEVELOPMENTAL COMPARISON TO DECIPHER PRIMING OF INDUCED DEFENSES IN MAIZE AND ITS EFFECTS ON A GENERALIST HERBIVORE

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ABSTRACT

Plants have a diverse array of defensive strategies that are induced by herbivory. Some of these defenses, such as herbivore-induced plant volatiles (HIPVs) are airborne and can be perceived by other plants. Priming is a strategy for plants to capitalize on their perception of HIPVs. The steps by which an undamaged plant becomes primed after perception of HIPVs are poorly understood, but the effects of priming are distinctive. Once an herbivore starts to feed, the primed plant displays a faster and stronger defense response. In maize, whether caterpillar feeding induces a primed defense response and how feeding on primed maize impacts the caterpillar remains relatively unknown. The defense profile consists of direct and indirect defenses. To characterize these defense categories, I assessed volatile production as a measure of indirect defenses, transcript levels of proteinase inhibitors as an estimate of direct defenses, and jasmonic acid (JA) levels as a general indicator of a plant defense response. To evaluate this, I performed a developmental comparison of two seedling stages, v1 with three leaves and v3 with five leaves. Here, I report that simulated and actual herbivory differentially elicit volatile production and proteinase inhibitor transcript accumulation in two maize seedling stages. Once the defense profiles from each developmental stage had been characterized, I proceeded to compare primed and non-primed defense profiles in v1 and v3 plants. To establish that the plants responded to the priming agent, a HIPV, I measured JA levels shortly after exposure to the HIPV. Both developmental stages contained elevated JA levels, which indicated that the plants responded to the priming treatment. Caterpillars feeding on primed v1 and v3 maize compared to non-primed maize did not elicit faster or higher amounts of volatiles within twenty four hours. In addition, JA levels were not higher in primed versus non-primed plants at several points during four hours of caterpillar feeding. However, bioassays indicated that the youngest-v1- primed maize plants reduced larval weights. In addition, primed v1 maize plants experienced less leaf

damage. Since the bioassays indicated that the primed v1 maize plants reduced caterpillar growth, I then assessed at a molecular level, whether transcripts of proteinase inhibitors were higher in primed plants. However, the transcripts at 6, 24, 48, and 72 hours were not higher in primed versus non-primed plants. These results suggest that priming in maize may be most effective at certain developmental stages. My findings provoke a re-assessment of defense characterization in primed maize. The ontogenic comparison reveals that different life stages of maize may prioritize different defense strategies and therefore early stages such as v1 can be expected to exhibit a stronger primed direct defense response. Later life stages such as v3 may shift their emphasis to indirect defenses. These findings deepen our knowledge of an agronomically important crop and are essential for the development of sustainable pest management strategies, which may act in concert with maize's own manifold defense system.

Key words: induced defenses, ontogeny, priming, *Zea mays*, *Spodoptera exigua*, herbivore-induced plant volatiles

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To my family, friends, and co-workers:

This would not have been possible without you. I thank you with all my heart.

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The plant-herbivore interface

Roots connect and imprison plants in their local environment. Their sedentary lifestyle prohibits them from re-locating to avoid disease, insect damage, drought, adverse weather, or growth-inhibiting soil conditions. In order to survive and reproduce, plants must respond and either tolerate or defend themselves from these multiple stresses. On the flip side, herbivores must cope with plant defenses to consume tissue for growth and reproduction. While some insects feed on many plant species, others feed exclusively on a few related species. Rather than assuming that all plants are nutritionally or preferentially equivalent, observations of herbivore preference for certain host plants accumulated. This led to two key ideas. One is that plants possess chemicals that are defensive [1,2]. And that plants and insects engage in a co-evolutionary arms race to minimize the negative effects of consumption [3]. Because these compounds did not appear to be involved in primary metabolic processes, they were termed secondary metabolites [4]. Understanding of plant defenses rapidly expanded to include not only

small molecules but anti-nutritive proteins, structural defenses, and volatiles and chemical rewards to attract natural enemies of the herbivore.

Regulation of defense expression: constitutive and induced defenses

In defense against herbivory, plants can continuously express and/or induce defenses once a threat is present [5,6,7,8]. Synthesis of toxic compounds uses resources that might otherwise be used for growth and reproduction. Thus, it is thought that these chemical defenses are costly and constitutive expression may only be to the plant's advantage when the threat of herbivory is high. In low risk environments, plants could reduce investment in defenses by waiting until a threat is present to induce defense production. This strategy does include the risk that the herbivore might consume a deleterious amount of tissue before defense induction is effective.

In many cases, plants constitutively produce low or moderate levels of a toxic metabolite but will produce greater amounts when herbivore damage occurs. For instance, parsnip (*Pastinaca sativa*) leaves maintain microgram quantities of furanocoumarins that double after cabbage looper feeding [9]. Defense expression is context- and cost-dependent. The parsnip webworm, a specialist herbivore, prefers to feed on the fruits. The parsnip fruits contain high constitutive levels of furanocoumarins that are not increased by artificial damage probably because of the high probability of attack and value for reproductive fitness [10]. Although plants can constantly produce high levels of defensive compounds, many plants boost their defenses only when herbivore damage occurs. In response to constitutive and induced plant defenses, herbivores employ counter-defenses by modifying their feeding behavior to avoid toxins [11,12,13], sequestering them to avoid toxicity and use as predation deterrents [14,15], and

detoxifying the metabolites [16,17,18,19,20]. Both constitutively and inductively regulated defenses can function either directly or indirectly defend the plant.

Functionality of defense expression: direct and indirect defenses

Direct plant defenses repress herbivore growth and survival by limiting consumption with structural defenses and toxins [21,22]. Direct defenses can have adverse affects on other trophic levels [23]. For instance, in tomato, induced plants significantly reduces parasitoid performance compared to un-induced plants [24]. In cucumber, spider mite feeding induces cucurbitacins that reduces susceptibility to spider mites on systemic leaves but increases susceptibility to a specialist beetle herbivore [25]. Aside from secondary metabolites (i.e. furanocoumarins, glucosinolates), plants contain a diverse array of plant defensive proteins including proteases [26,27], lectins [28,29], chitinases [30], oxidative enzymes [31,32,33,34], amino-acid degrading enzymes [35,36], and proteinase inhibitors (PI) [37,38,39,40,41,42]. Recently, identification of plant defensive proteins has leapt forward because of protein identifications from insect frass and digestive fluids [34,43]. In order to interact defensively, these proteins must remain stable throughout the insect gut environment and thus often remain in the frass [44]. Plants contain two major PI types that inactivate cysteine (i.e. cystatins, papain) and serine proteases (i.e. trypsin, chymotrypsin) [45,46,47,48,49]. These two types can act synergistically with each other or with other toxins [50,51,52]. Insects defend their own digestive systems by countering with compensatory consumption and expression of resistant proteases [38,39,53,54,55,56,57]. And in response, plants appear to counter by evolving novel PIs [58,59].

A plant may indirectly defend itself against herbivores through the production of volatiles or extrafloral nectaries to attract predators and parasitoids of the herbivore [60]. Plants produce a broad spectrum of volatile compounds in response to abiotic and biotic stresses [61,62,63,64,65].

Historically, researchers focused on two main questions, one chemical in nature and the other ecological.

What compounds do plants release into the atmosphere?

What function do these compounds have?

The role of plant volatiles in direct and indirect defense

Typically, herbivore-damaged plants produce >20 compounds which fall into several biosynthetic/functional groups including: green leaf volatiles (GLVs), monoterpenes, sesquiterpenes, homoterpenes, and aromatic compounds. GLVs, a blend of C_6 and derivative compounds, are the widely recognizable wound-induced compounds i.e. the smell of freshly mown grass [66,67,68]. In general, volatile production is light dependent with amounts substantially decreased during the night. However, GLVs demonstrate the reverse pattern with release occurring in the highest quantities during the night [69,70,71]. Qualitatively and quantitatively, the blend shifts from plant species to plant species and in some cases from variety to variety within a species [72].

Plant volatiles fall into two broad functional categories- development and defense. For instance, extensive research has established the role of ethylene in many developmental processes including fruit ripening, senescence, leaf expansion, flowering, and abscission [73,74]. Plants use volatiles for direct and indirect defense against pathogens and herbivores [75,76]. In other bitrophic contexts, plant volatiles reduce fungal growth or infection and attract pollinators[77]. During herbivory, plants emit a blend of volatiles specific to the herbivore [78,79]. This odor blend attracts parasitoid wasps which parasitize the herbivore [80]. Volatiles produced in response to herbivory deter oviposition by herbivores [70], and induce plant defense responses [81]. The plant-herbivore-parasitoid interaction is an example of a tritrophic interaction that is

predominately a volatile-mediated process [80,82,83,84,85,86]. Moving to belowground, herbivore-damaged maize roots release *(E)*-β-caryophyllene, a sesquiterpene, to attract entomophagous nematodes [87,88,89]. Variations on the theme occur during oviposition-induced blends attracting egg parasitoids [90,91] and enhancing extrafloral nectary secretion in a plant-herbivore-predator interaction [92]. Not only do plant volatiles transfer information between trophic levels but accumulating evidence suggests that this also occurs between and within plants [71,92,93,94].

Elicitation of plant defenses by herbivores

The plant defense response to herbivory has been well-characterized across plant species and insect feeding habits. However, at a mechanistic level, elicitation of plant defenses by herbivores is poorly understood. Behaviorial, mechanical, and chemical processes occur during herbivore feeding at varying spatial and temporal scales. Separation of effects due to the mechanical versus chemical nature of herbivore feeding is fraught with difficulty. Indeed, elaborate attempts have been made to replicate the mechanical nature of caterpillar feeding [95,96]. Mithofer and colleagues (2005) used a mechanical device to simulate caterpillar feeding. The MecWorm, a robotic caterpillar-simulacrum, elicited lima bean to produce a qualitatively but not quantitatively similar volatile profile. Transcript profiling experiments comparing mechanical damage and either elicitor application or herbivory, did reveal herbivore-specific transcripts [97,98,99,100,101,102]. Another technique manipulates the structures that deposit oral secretions, the spinnerets, which are ablated allowing the caterpillar to feed without oral secretions [103]. This technique is relatively straight-forward and accessible compared to the MecWorm. Aside from the mechanical nature, significant steps toward understanding the chemical nature of oral secretions have been made.

During pathogen infection, plants perceive elicitors associated with the pathogen, otherwise known as microbe-associated molecular patterns (MAMPs) [104]. Similar to MAMPs, it appears that plants respond to herbivore-associated molecular patterns (HAMPs) [105]. A step towards understanding HAMPs has been the isolation of elicitors from herbivore oral secretions (OS) and the putative identification of a receptor for one of these elicitors [106]. One of the first demonstrations of an herbivore-derived elicitor of plant volatiles was an enzyme, β-glucosidase, present in the regurgitant of *Pieris brassicae* caterpillars [107]. Thus far, the majority of isolated elicitors are fatty-acid derived or small protein fragments [43,108,109]. The fatty-acid derived elicitors appear to be widespread through the Lepidoptera, Orthoptera, and Diptera [110,111,112]. Most of these compounds have been characterized as elicitors of plant volatile production. In a survey of plant species including maize, soybean, eggplant, cowpea, and Arabidopsis, the elicitors displayed differential activity with regards to phytohormone and volatile induction [113]. One elicitor, volicitin (a fatty acid amino conjugate) was shown to be deposited onto leaves using a radioactivity assay [114]. However, the type and amount of OS applied to these wounds may not represent that of the feeding herbivore [115]. Alternatively, defense induction may result from the plant recognizing its own cellular debris during tissue damage [116]. Not all components of OS elicit plant defenses, for instance, glucose oxidase in *Helicoverpa zea* OS suppresses plant defense responses [117,118].

Priming of induced resistance

Inducible defenses allow plants to conserve energy by waiting until an herbivore attacks to begin production of costly defenses. However, in the delay between the beginning of herbivore feeding and activation of the plant's defenses, the plant may lose a considerable amount of tissue. Priming allows a plant to use environmental cues to pre-warn or prime their defense

responses to react with a stronger, faster response when herbivory actually occurs. To trigger the primed state, an undamaged plant is exposed to a priming agent. After initiating the primed state, a primed plant responds to a stress elicitation with stronger, faster defense or tolerance [119]. This phenomenon is not limited to plant-insect interactions but can also apply to plant-pathogen interactions and abiotic stresses [120]. In addition, priming can occur between individual plants or within a single plant, where application of stress to one part of the plant can cause priming of other parts of the plant. The key questions include:

How is a plant primed?

When a primed plant is elicited, how does the plant respond?

How is the attacking organism affected?

Does priming benefit the plant? How and when does it benefit the plant?

To lay the research foundation of this new and emerging topic, first, I will address chemically-mediated priming with an emphasis on plant-pathogen systems because the first demonstration of priming occurred here along with most of the advances in understanding priming at a molecular level. Subsequently, I will move into a discussion of volatile-mediated priming, the subject of this dissertation.

Chemically-mediated priming

Most of the literature concerning plant-pathogen and abiotic stress interactions, utilizes either a non-pathogenic organism or a synthetic chemical to induce the primed state and functions within an individual plant [121,122]. The first discovery and recognition of priming occurred when parsley (*Petroselinum crispum*) suspension cultures primed with methyl jasmonate or salicylic acid analogues secreted five to thirteen fold higher levels of coumarins (defensive compounds) after fungal elicitation compared to non-primed, elicited controls [123]. In plant-

pathogen systems, induced resistance occurs via systemic acquired resistance (SAR), induced systemic resistance (ISR), and β-aminobutyric acid-induced resistance (BABA-IR). SAR is an immune response to a primary infection. Typically, when an avirulent pathogen triggers SAR, then the plant is immune to secondary infection [124]. The onset of SAR is commonly associated with the expression of pathogenesis-related (PR) proteins [125]. In general, priming of SAR is thought to be dependent on the phytohormone salicylic acid (SA) and regulated in part by the NPR1 gene [126,127,128]. In contrast, ISR does not rely on SA but utilizes two other phytohormones, ethylene and jasmonic acid. Primed resistance due to BABA-IR appears to involve both SA and NPR1 dependent and independent effects [122,129,130,131]. Both SAR and ISR occur in nature, however, BABA-IR is a chemically-induced form of resistance used to understand mechanisms of induced resistance.

Plants exposed to β-aminobutyric acid, a non-protein amino acid, display enhanced resistance to an array of biotic and abiotic stresses [122,132]. Aradopsis mutants impaired in BABA-IR were identified by screening, at high application rates, for resistance to BABA-induced sterility. To evaluate which stress response pathways were affected in the mutants, each was challenged with two pathogen types and salt stress. This led to the identification of three key regulators of priming: a cyclin-dependent kinase-like protein, a zeaxanthin epoxidase, and a phosphoinositide phosphatase. Interestingly, none of these mutations abolished resistance to all three stresses. Previously, a novel mutant, *edr1,* was found to possess enhanced disease resistance [133]. Subsequent work identified the edr1 mutation to encode a MAPKK kinase [134]. MAP kinases have been implicated in multiple stress responses including herbivory [135,136,137,138]. This mutant differed from other disease resistant mutants because it did not constitutively express pathogenesis-related proteins but had a stronger, faster defense response once challenged. The following example uses the *edr1* mutant to examine the costs of priming in Arabidopsis.

To estimate the fitness of primed Arabidopsis plants, relative growth rate and seed production were measured for both chemically-induced and mutant lines with and without disease pressure. Depending upon the concentration applied, BABA can prime or directly induce defenses in *Arabidopsis thaliana* [139]. Early efforts at characterizing BABA-IR were often hampered by the use of concentrations toxic to plants [140]. To compare the costs of BABA-IR to prime or induce direct defense, Arabidopsis plants were treated at various concentrations with BABA and benzothiadiazole (BTH), an inducer of SAR [139,141]. These plants were then either exposed to a pathogen or remained un-infected. Relative growth rate and seed production was found to be lower in plants treated with direct-defense inducing concentrations of BABA and BTH compared to primed and control plants. This finding was not affected by toxicity of BABA and BTH because treatment of a mutant, *npr1-1*, impaired NPR1-dependent disease resistance, did not exhibit lower seed set or relative growth rate [142,143]. In addition, this suggests that BABA- and BTH-induced direct defenses are responsible for the observed reduction in fitness. In Arabidopsis, the *edr1* mutant, unlike other disease-resistance mutants, is constitutively primed for pathogen resistance [133,134,144]. In a comparison of *edr1* and *cpr1*, a constitutively defended mutant, relative growth rate and seed production was negatively effected in the *cpr1* but not *edr1* compared to wild-type controls. In fact, the constitutively primed mutant, *edr1*, appears to achieve higher fitness when under disease pressure compared to wild-type and plants constitutively expressing direct defenses.

Very little research has addressed chemically-induced priming in plant-herbivore interactions. However, in poplar, *Populus*, external application of a cytokinin solution primed saplings that when infested with gypsy moth larvae, *Lymantria dispar*, produced elevated levels of linolenic acid, jasmonic acid, and defense-related transcripts [145]. Herbivore fitness was also reduced at the larval stage when feeding on primed, wounded developing leaves, however, no control for cytokinin application was included. Recent plant-herbivore research is now bridging

belowground and aboveground interactions and addressing whether belowground herbivory may prime vegetative tissues [146,147,148]. In maize seedlings, priming via a soil drench of abscisic acid and subsequent infestation with *Spodoptera littoralis* caterpillars led to enhanced levels of chlorogenic acid in the leaves [149]. This is similar to the effect of maize roots infested western corn rootworm, *Diabrotica virgifera* [150]. However, *S. littoralis* feeding on leaves of *D. virgifera*-infested maize experienced reduced growth but this was not observed for abscisic acid-primed seedlings. This suggests that elicited, primed chlorogenic acid is not responsible for herbivore resistance or that resistance may require other factors. In contrast to plant-pathogen studies, most plant-herbivore studies have examined the effects of volatile-mediated priming.

Volatile-mediated priming

Volatiles can prime within and between plants. Several challenges occur when attempting to establish that volatiles are priming agents. Can these compounds act as signals? Unfortunately, no receptor and signal transduction system has been identified for most of the herbivore-induced plant volatiles with the exception of ethylene [151]. As an alternative to identifying a receptor-signaling system, the ability of these volatiles to induce biochemical alterations in plants has been used to establish their biological activity. In general, gene expression changes and ethylene release have been associated with exposure to green leaf volatiles (GLVs) [152,153,154,155,156,157], cis-jasmone [85,86], monoterpenes [158], and homoterpenes [159]. Spatially, volatiles could function between leaves and between plants, however, the effectiveness appears to be limited by distance [160]. Leaves or branches within a plant may "eavesdrop" using volatiles when signaling is not effective because of limited vascular connections [161,162,163]. Indeed, plants responding to local herbivore damage will emit

volatiles from undamaged, systemic leaves [164,165,166,167]. This suggests that plants may overcome vascular constraints using volatiles to signal systemically to the entire plant.

Within-plant priming

The effect of vascular constraints and defense responses to herbivory in poplar is well established [168,169,170]. Because defense expression is known to depend on the vascular connectivity, it is an ideal system to explore whether volatile-mediated priming functions in unconnected leaves. When elicited with gypsy moth (*Lymantria dispar)* feeding, primed poplar (*Populus deltoides x nigra*) leaves emit more volatiles and contain higher cis-jasmonic acid, linolenic acid, and defense-related transcripts [171,172]. Jasmonic acid (JA) is implicated in many physiological and defensive functions including anther dehiscence, pollen development, tuberization, senescence, flowering, drought, UV light, wounding, necrotrophic pathogens, and insects [173]. In response to insect feeding, elevated JA levels correlate with the induction of direct and indirect defenses. Linolenic acid is the starting material for JA synthesis [174]. Expression of a serine proteinase inhibitor was higher in primed poplar leaves after 24 hours of feeding but not a marker of condensed tannin synthesis and two volatile synthesis related genes. Leaf consumption by gypsy moth larvae was not different between primed and control treatments.

In another example of priming overcoming vascular constraints, blueberry (*Vaccinium corymbosum),* herbivore-induced plant volatile (HIPV)-primed branches experienced less leaf consumption by gypsy moth larvae and produced more volatiles normalized by leaf area damaged [175]. After 3 days of exposure to a caterpillar-induced volatile blend, leaves contained higher levels of cis-jasmonic acid but not salicylic acid or linolenic acid. In lima bean, *Phaseolus lunatus*, HIPV-exposed tendrils in the field experienced less herbivore damage and had an increased number of leaves and leaf shoots compared to un-exposed tendrils [92,176]. Upon

elicitation with mechanical damage, extra-floral nectary (EFN) secretion increased in HIPV-exposed lima bean tendrils compared to elicited, un-exposed controls. Lima bean plants utilize EFN secretion to indirectly defend themselves by attracting ants that reduce herbivore pressure [177]. EFN accumulation can be triggered by exposure to HIPVs [178]. Interestingly, volatiles may also play a role in priming for pathogen resistance. In lima bean, *Phaseolus lunatus*, SAR-induced plants emitted volatiles that primed neighboring plants [179]. Once these primed neighbors were challenged with a virulent strain of *P. syringae*, these plants had higher transcript levels of pathogenesis-related 2 (PR-2) but not lipoxygenase. Within-plant priming is not restricted to indirect defenses but may also involve direct defenses. However, the effects upon herbivore performance remain to be established.

Between-plant priming

Recent research suggests that priming may explain how undamaged plants react to signals from damaged neighbors. Between plant signaling to induce herbivore resistance has been a controversial subject since the first report [180]. Experimental design, location (i.e. field or laboratory), and inconsistent findings drew criticism and skepticism regarding the phenomenon [181,182]. Because the mechanism was unknown, a contributing factor to the contradictory results was analysis for herbivore resistance traits before herbivore damage. This is not surprising when the cost of producing defense(s) without the presence of an herbivore can outweigh the benefits. An alternative explanation emerged that perhaps, plants primed their defenses for a faster, stronger response once an herbivore begins to feed [183]. The receiver plant or leaf does not induce a resistance trait at the time of stimulus perception but in fact waits until herbivory begins to initiate a stronger, faster response.

The first report of volatile-mediated priming in a plant-herbivore system occurred when GLV-exposed 'Delprim' maize (*Zea mays)* plants contained higher jasmonic acid levels and emitted more volatiles after simulated herbivory compared to elicited, un-exposed control plants [71]. To verify that plants responded to GLV-exposure, plants were exposed to synthetic GLV compounds and herbivore-induced plant volatiles (HIPVs) for a brief period. Jasmonic acid (JA) levels were found to be higher in exposed versus unexposed plants. For elicitation, herbivory was simulated by mechanically damaging the leaf surface with a razor blade and adding an aliquot of beet armyworm (*Spodoptera exigua)* regurgitant. Elicited, primed plants produced more JA at thirty and sixty minutes after simulated herbivory treatment. In addition, elicited, GLV-exposed plants produced more total volatiles at four and five hours after elicitation, which plateaued one to two hours later. Attenuation of volatile production may have occurred because simulated herbivory occurred as a single elicitation and not repeated, continuous stimulations experienced during caterpillar feeding.

In a separate study, elicited, primed 'Delprim' maize seedlings contained higher defense-related transcripts, emitted more volatiles, attracted more parasitoids, and reduced larval weights compared to elicited, un-primed control plants [184]. A cDNA library was generated for herbivore-damaged maize seedlings to identify a subset of herbivory-specific genes that were then profiled 3, 6, and 9 hours after simulated herbivory and JA application. Subsequently, these genes were used to test for higher gene expression in elicited, primed compared to elicited, un-primed control seedlings. Of these ten genes, six were "priming-responsive" and four were "priming non-responsive" in primed plants elicited with simulated herbivory. Several of these "priming-responsive" genes were annotated as proteinase inhibitors (PIs). Typically, plants contain higher PI transcript levels or PI activity during herbivory and these are associated with reduced caterpillar performance and survival [185,186]. In a larval bioassay, *Spodoptera*

littoralis larval weight gain was reduced after eleven hours of feeding but was "less-pronounced" at later time points (data not shown in paper).

In another study, field and lab experiments were performed to identify elicited, primed direct defenses and their effects on a specialist herbivore. Damaged wild sagebrush (*Artemisia tridentata)* produces volatiles that signal to adjacent tobacco (*Nicotiana attenuata)* plants causing the tobacco plants to prime their defenses [183]. Tobacco responsiveness to sagebrush volatiles theoretically would be advantageous to tobacco if the herbivore community was shared and likely to migrate from sagebrush to tobacco. However, in nature, sagebrush and tobacco rarely grow within an effective distance of each other for volatile signaling to occur. Also, the common herbivore between sagebrush and tobacco, the grasshopper, causes relatively minor damage on tobacco at their study sites. Interestingly, despite the unlikely nature, this study does demonstrate that volatile-mediated priming occurs between sagebrush and tobacco and reduces herbivore pressure.

In a field experiment, no feeding damage was observed on primed tobacco plants. When artificially infested with *Manduca sexta,* larval mortality was higher on primed tobacco compared to un-primed tobacco plants. However, egg predation rates did not differ between treatments. Priming, via exposure to sagebrush volatiles, induced trypsin proteinase inhibitor (TPI) transcripts compared to controls. However, when these primed plants were elicited, TPI gene expression did not differ, although, the onset of TPI activity was faster but not greater in elicited, primed plants. Exposure to clipped sagebrush volatiles in lab and field-grown tobacco led to a down-regulation of photosynthesis-related genes. In general, herbivory and other biotic stresses are associated with suppression of photosynthesis indicating a shift away from growth towards defense [102,187,188,189]. Clipped sagebrush released a complex blend of volatiles including a green leaf volatile, methacrolein, monoterpenes, sesquiterpenes, and several unknown compounds. Two of these compounds, *(E)*-2-hexenal and methacrolein, were tested individually.

Tobacco primed with these compounds exhibited higher trypsin proteinase inhibitory activity compared to elicted, un-exposed control plants. Tobacco plants primed by exposure to clipped sagebrush volatiles experienced less herbivore damage and had higher direct defense expression.

In HIPV-primed cabbage (*Brassica oleracea)* plants, both indirect and direct defenses were higher compared to unprimed plants [190]. HIPV-primed plants contained higher transcript levels of lipoxygenase compared to un-primed plants. In addition, lipoxygenase transcripts were elevated in elicited, primed plants compared to elicited, un-primed control plants. Lipoxygenase reacts with linolenic acid to produce a hydroperoxy fatty acid that is a starting substrate for various oxylipins including jasmonic acid [191]. Also, lipoxgenase ingestion can reduce herbivore performance [32]. Larval weights of a specialist herbivore (*Pieris brassicae)* and a generalist herbivore (*Mamestra brassicae)* were reduced on elicited, primed plants after seven and ten days of feeding at an infestation level of five neonates but not ten. Attraction of naïve parasitoid wasps (*Cotesia glomerata)* was higher for elicited, primed compared to elicited, un-primed control plants. Herbivory induces primed cabbage plants to exhibit a heightened direct and indirect defense response and this can reduce herbivore performance.

In summary, understanding how eliciting a primed plant affects the herbivore will provide context for investigating the defense response of primed plants. Relatively little is known about the mechanisms of signaling to initiate the primed state and, at a molecular level, the elicited defense response. The practical applicability is enhanced by characterizing this phenomenon in a plant of agronomic importance. The majority of interplant signaling and priming studies have examined woody perennial tree species and wild herbaceous annuals but not cultivated species. Maize is an ideal study organism for interplant priming. For instance, at early infestations, caterpillar feeding tends to be isolated in the whorl, the site of developing leaves. This limits the size of the headspace. Also, maize, either in small subsistence plots or vast

agribusiness fields, is grown with minimal spacing that maximizes the likelihood that volatiles will travel between plants [160].

Defense in a developmental context

Plants rely on the surrounding environment for sunlight, soil nutrients, and water. As part of this sessile life history, plants must cope with and defend themselves from a variety of abiotic and biotic stresses. Throughout a plant's life, it must allocate resources effectively to growth, defense, and reproduction [192,193]. Despite a paucity of research and appreciation of developmental effects on resource allocation, ontogeny significantly affects a plant's expression of traits [194,195]. Many theories predict how environmental factors affect plant defense expression, growth, and differentiation [196]. However, at this point, a single theory has not adequately extended to all studied systems [197].

The importance of changing patterns of defense expression during a plant's life is often ignored but is an important facet of selection for defensive traits [198]. Genotypic variation appears to influence resistance at certain developmental stages when measured by herbivore damage in wild gourd, *Cucurbita pepo* subsp. *texana* [199]. Alternatively, in *Plantago lanceolata,* leaf and plant age can account for more of the variation in a plant defensive compound compared to genotype [200]. In terms of defense in a developmental context, a meta-analysis revealed that no clear ontogenic patterns exist for defense expression across the plant kingdom [201]. Ontogenic patterns of defense expression varied by the type of plant life form (i.e. tree or herb), herbivore, and defensive traits, including tolerance as a defensive trait [201]. In addition to ontogenic variation in defenses, herbivore preference and community structure can change during a plant's development [202,203,204]. The plant's ontogenic stage and life form

(i.e. tree or herb) may affect whether it attempts to either resist herbivory by defensive measures or to tolerate herbivory via compensatory growth [205,206,207,208,209].

Plant developmental stages can differentially express direct and indirect defenses. Functionally, direct defenses operate to reduce herbivore performance with toxins and/or reduce tissue consumption with antifeedants and physical barriers. Indirect defenses attract natural enemies of the herbivore. The following will discuss constitutive and induced direct and indirect defenses in herbaceous plants according to developmental stage.

At germination, plants can have increased direct defenses and reduce herbivore performance compared to later seedling stages. However, the opposite may occur depending on the measured defensive trait and plant species. In a comparison of high and low-cucurbitacin containing *Cucumis sativa*, *Spodoptera exigua* larvae performance was enhanced on true leaves, which contain lower constitutive levels of curcurbitacins, compared to cotyledons [25]. In *Brassica juncea* seedlings, *Spodoptera eridania* larval performance increased with increasing seedling ages, this was correlated to declining activity of myrosinase, the enzyme responsible for converting glucosinolates into the much more toxic isothiocyanates [210]. Proteinase inhibitor (PI) inducibility declined in tomato plants as they aged [211]. However, in a field experiment, wounding induced PI production at mature stages, although, not in systemic tissues as the plants aged [212]. Conversely, growth of *Manduca sexta* larvae was reduced when fed on 4 week old, with lower PI content, compared to 2 week old plants [213]. Constitutive expression of phenolics, alkaloids, and cyanogenic glycosides increased with age in six of seven dicotyledenous grassland species during seedling development [214].

In comparisons of vegetative and reproductive stages, constitutive and induced direct defenses tend to shift from leaves to reproductive stages once flowering begins. Wild parsnip (*Pastinaca sativa*) produces constitutive levels of total furanocoumarins at all stages but the highest level occurs at the reproductive stage and is located in the reproductive structures [215].

In wild tobacco (*Nicotiana attenuata),* both constitutive and induced expression of direct defenses change depending on the developmental stage. These plants respond to methyl jasmonate, a potent defense elicitor, by increasing proteinase inhibitor content at the rosette stage but not the reproductive stages [216]. However, the reproductive stages maintain constitutive levels of proteinase inhibitors. Similarly, leaf damage increases total nicotine for the whole plant at the rosette stage but not the reproductive stages [217].

In another study, rosette stage plants contained constitutively higher amounts of caffeoylputrescine that declined in the leaves and shifted to buds and flowers at the reproductive stage [218]. To determine whether this phenylpropanoid was involved in both defensive and developmental processes, bioassays were performed to assess herbivore performance and plant fitness. Seed production did not differ in plants of two genotypes, the wild-type and a mutant that did not express a key regulator of caffeoylputrescine and dicaffeoylspermidine synthesis. This suggests that these phenylpropanoids were not directly involved in reproduction. Performance of a generalist and a specialist herbivore was higher when feeding on the mutant lacking phenylpropanoids confirming the defensive function of these compounds. Additionally, constitutive levels of 17-hydroxygeranyllinalool diterpene glycosides, a class of defensive compounds, increase in wild tobacco plants from the rosette to flowering stages [219].

Aside from direct defenses, very little is known about the ontogenic specificity of indirect defense expression. Within the same plant stage, young cucumber leaves infested with spider-mites attracted more predators compared to older infested leaves [220]. In a comparison of vegetative and reproductive stages of soybean (*Glycine max*), *Spodoptera frugiperda* caterpillars induced ten times the amount of total volatiles when feeding on a pre-reproductive plant [221]. The difference in volatile induction was not due to differences in leaf consumption. In addition, constitutive volatile emission was higher in the vegetative compared to the reproductive plants. Similarly, in a desert shrub, *Datura wrightii*, volatiles were elevated early in the season during the

vegetative stages [222]. This shrub experiences herbivore damage throughout the season. To control for the effects of decreasing humidity and water availability as the dry season continued, reproductive stage plants were clipped to promote resurgence of vegetative growth [61]. In these "rejuvenated" plants, volatile emissions were somewhat restored. In maize, sesquiterpene induction is greater in vegetative compared to reproductive stages [223]. Interplant communication may also be dependent upon the developmental stage. In a perennial desert shrub, *Artemisia tridentata*, plants exposed to young emitter plants experienced less herbivore damage compared to old emitter plants [194]. Conversely, young receiver plants experienced less herbivore damaged compared to old receiver plants. No difference in herbivore preference was observed for unexposed young and old control plants.

Maize defenses

Maize provides a unique opportunity to study developmentally specific defense expression due to its morphologically well-defined development [224]. Most laboratory and field research in maize has focused upon a single developmental stage. Laboratory assays of volatile induction in maize due to herbivory have been characterized at early seedling stages, in most cases, fourteen days after seeding. In contrast, much of the field research concerns biotic and abiotic factors of yield reduction at the economically important reproductive stages. Observations of altered herbivore preference or performance between developmental stages do exist. For example, infestations of European corn borer (*Ostrinia nubilalis)* at early vegetative stages result in low survivorship of ECB larvae [225,226]. Additionally, pathogen resistance traits may be developmentally dependent. In the *Cg1* maize mutant, the juvenile to adult vegetative transition is prolonged. Infection by common rust, *Puccinia sorghi*, was lower in wild type older (mid-whorl) leaves compared to wild type young (seedling), *Cg1* young, and *Cg1* older

leaves [227]. Yet, how maize expresses both direct and indirect defenses during its development is relatively unknown.

Despite its importance as an agronomic crop, the suite of maize direct defenses in response to insect feeding remains relatively unexplored with a few notable exceptions [228,229]. Maize direct defenses include chemical and protein toxins and structural modifications. Benzoxazinoids or cyclic hydroxamic acids are widely distributed through the Poaceae, the grass family [230]. Recently, genes regulating their biosynthetic enzymes have been identified in maize [231,232,233]. Constitutive expression occurs in a developmentally specific manner in maize and wheat with concentrations that increase shortly after germination and decline in later seedling stages [234,235,236]. However, this is not necessarily the case with other grasses, such as *Arundo donax*, a subtropical perennial reed-type grass that varies in concentrations of DIMBOA, a benzoxazinoid, throughout the season with the highest amounts occurring at the end of the season [230]. Aside from constitutive levels, these compounds can also be induced by herbivory [150,237,238] and are associated with resistance to European corn borer, and two armyworm species, *Spodoptera eridania* and *S. exigua* [239,240,241,242]. A recent study reported that gut homogenates from three *Ostrinia* spp. differentially degraded cyclic hydroxamic acids in their diet [243]. The species with the highest degrading activity also had the highest larval performance on diets containing cyclic hydroxamic acids. In addition to the benzoxazinoids, maize contains another resistance factor, maysin, a flavone glycoside. Maysin occurs in the silks surrounding the ear that is encased in the husk. Despite initial reports of maysin reducing *Helicoverpa zea* larval performance, field tests of high and low maysin genotypes found no correlation between maysin content and reduction in ear damage [244,245,246,247,248].

Maize also produces defensive proteins including inducible proteinase inhibitors (PI) and proteases. Wounding, insect feeding, simulated herbivory, and exposure to herbivore induced

plant volatiles can elicit the expression of PIs [249,250,251]. In addition, some maize genotypes contain a cysteine protease that degrades the gut lining or peritrophic matrix of Lepidoptera larvae [27,228,229,252].

Structural defenses including silica, trichomes, and leaf toughness may play an important role in maize resistance to herbivores. In the grass family, many species contain silica that increases after grazing and is thought to negatively affect mouthpart structures and digestion [253,254]. In an survey of Southwestern corn borer-(*Dietraea grandiosella*) resistant and -susceptible maize genotypes, silica content was similar [255]. Trichomes on maize leaves have been implicated in the arrestment and searching time of parasitoids and inhibition of oviposition by an herbivore [256,257]. In a study of trichome initiation, treatment of maize with jasmonic acid or cytokinin increased the total number of trichomes including stinging hairs [258]. Leaf toughness, in particular hemiceullulose content, may play a significant role in resistance to several Lepidopteran herbivores [255,259,260].

In contrast to maize direct defenses, the induction and release of herbivore induced plant volatiles (HIPVs), an indirect defense, is well characterized [261]. HIPVs indirectly defend maize by deterring aphid colonization [262] and by attracting parasitoid wasps above-ground [80,84,263,264,265] and entomophagous nematodes below-ground [87,88,89]. The biosynthesis is well-understood compared to other plant species including the synthesis of sesquiterpenes [88,266,267,268,269], monoterpenes [270], homoterpenes [271], and indole [272,273]. Volatiles may be induced by mechanical damage and application of herbivore oral secretions [264]. An elicitor of HIPVs was identified from the regurgitant of *Spodoptera exigua* caterpillars [109]. Induction of volatiles appears to depend on herbivore feeding type (i.e. chewing, piercing/sucking) [274] and are influenced by abiotic factors [61].

Chapter 2

Maize developmental stage affects indirect and direct defense expression

Abstract

Typically, indirect and direct defense expression is characterized for a plant at a single developmental stage. Although, considerable research has addressed defenses within a developmental context for trees, little is known about annual plants, in particular, one of agronomic importance. Here we report the characterization of direct and indirect defense profiles for two seedling stages of maize, v1 and v3. In maize, herbivore induced volatiles indirectly protect the plant by attracting parasitoids of the herbivore. Volatile profiles during simulated and actual herbivory were measured for v1 and v3 plants. Simulated herbivory did not induce total volatile production in the youngest seedling stage, v1. During caterpillar feeding, both seedling stages produced a similar amount of total volatiles when normalized to fresh weight, but produced a qualitatively different blend. Herbivory elevated transcript levels of a sesquiterpene biosynthetic gene, farnesyl pyrophosphate synthetase, FppS, in both stages but the induction was less in v1 than v3. Jasmonic acid levels were evaluated after simulated herbivory. Both regurgitant-treated v1 and v3 plants contained elevated levels of jasmonic acid. This indicated that v1 plants do respond to herbivory. To assess direct defense expression, transcript measurements of four proteinase inhibitors were performed using quantitative PCR. Expression of a cystatin-like proteinase inhibitor is higher in v1 caterpillar-fed plants. To assess beet armyworm, *Spodoptera exigua,* larval performance, bioassays were performed with intact v1 and v3 plants. Total larval mass was reduced on v1 compared to v3 plants. V1 plants produce a limited amount of volatiles, typically an indirect defense, and engage in a direct defense response.

These experiments show that indirect and direct defense expression, in response to regurgitant application and herbivory, are affected by the plant's developmental stage.

Introduction

Plants rely on the surrounding environment for sunlight, soil nutrients, and water. As part of this sessile, opportunistic life history, plants must cope with and defend themselves from a variety of abiotic and biotic stresses. Generally, a plant's life cycle is viewed as a balancing act of trade-offs; a game of allocation between growth, reproduction, and defense [23]. Allocation implies that there are costs to each function. The existence of a cost to defense coincides with the observation that not all genotypes exhibit equivalent resistance to insect feeding. Herbivore resistance traits vary widely across the plant kingdom. These defense responses are broadly characterized according to their regulation: induced and constitutive and their function: direct and indirect.

In defense against herbivory, plants have two strategies, to wait and produce defensive materials once an herbivore begins to feed or to continuously express these defenses. Both induced and constitutively-regulated defenses can function either directly or indirectly to reduce herbivore pressure. Direct defenses negatively impact the herbivore via anti-nutritive, anti-digestive, or repellent mechanisms [5,185]. For example, plants produce proteinase inhibitors that alter protease activity in herbivore digestive tracts [275]. This can limit larval growth that can benefit the plant [52]. An indirect defense operates by attracting predators and parasitoids of the invading herbivore. Herbivore induced plant volatiles can function to attract parasitoids and predators [80,276,277], repel ovipositing moths [70], and signal within and between plants [92,183].

Plants use volatiles for direct and indirect defense against pathogens and herbivores [75,76]. Plants produce a broad spectrum of volatile compounds in response to abiotic and biotic stresses [61,63,64]. Plant volatiles fall into two broad functional categories: development and defense. For instance, extensive research has established the role of ethylene in many developmental processes including fruit ripening, senescence, leaf expansion, flowering, and abscission [73,74]. During herbivory, plants emit a blend of volatiles specific to the attacking herbivore [78]. This odor blend attracts parasitoid wasps, which then parasitize the herbivore [80]. An herbivore's regurgitant or oral secretion when applied to a mechanically damaged plant results in a similar volatile profile [264].

Throughout a plant's life, it must allocate resources effectively to growth, defense, and reproduction. Additionally, depending on the life history stage, the types and intensity of defense expression during herbivory is expected to change [202]. There is no clear direction of plant defense expression and age when considering the plant kingdom [202]. Much of the literature has focused on evaluating defenses on different leaf morphological types within a developmental stage. This provides a basis for how a plant will alter its investment in leaf types and reproductive structures. Older leaves of *Nicotiana attenuata* contain lower levels of caterpillar regurgitant-induced transcripts [278]. Younger lima bean leaves infested with spider-mites produce a volatile blend that attracts more predators [220]. In a comparison of developmental stages, jasmonic acid application induced nicotine accumulation in rosette stage but not flowering stage tobacco plants [217]. Proteinase inhibitor inducibility declined in tomato plants as they aged [211]. Maize provides a unique opportunity to study developmentally specific defense expression due to its morphologically well-defined development [224].

Most laboratory and field research in maize has focused upon a single developmental stage. Despite its importance as an agronomic crop, the suite of maize direct defenses in response to insect feeding remains relatively unexplored with a few notable exceptions [228,229]. In

comparison, the maize indirect defense profile is more complete. For instance, volatile induction by caterpillar feeding is partially due to fatty acid amino acid conjugates present in the caterpillar's regurgitant [264]. In addition, the herbivore-induced volatile blend is known to contain green leaf volatiles, terpenes, and aromatic compounds. From a developmental perspective, herbivore infestation induces qualitatively and quantitatively different sesquiterpene profiles according to maize vegetative or reproductive stage [223]. Laboratory assays of volatile induction in maize due to herbivory have been characterized at early seedling stages, in most cases, fourteen days after seeding. In contrast, much of the field research concerns biotic and abiotic factors of yield reduction at the economically important reproductive stages. Yet, how maize expresses both direct and indirect defenses during its development is relatively unknown. Observations of altered herbivore preference or performance between developmental stages do exist. For example, infestations of European corn borer larvae, *Ostrinia nubilalis,* at early vegetative stages result in low survivorship of ECB larvae [225,226]. At a molecular level, maize contains toxic hydroxamic acids, notably DIMBOA, that are constitutively higher in young seedlings [230]. It could be assumed that young maize plants rely on constitutive direct defenses. However, they may actively defend themselves against herbivores.

In this report, we investigated whether v1 and v3 plants differentially express indirect and direct defenses during herbivory. A maize plant matures through a series of morphologically distinct vegetative and reproductive stages [224]. On average, maize reaches the v1 stage 10 days post-seeding and the v3 stage 14 days post seeding (Fig. 1). Contrary to expectations, headspace sampling of v1 and v3 regurgitant-treated plants suggested that v1 plants do not exhibit an herbivore-specific response. To evaluate whether v1 plants do not respond to herbivory, we assayed for jasmonic acid production in response to caterpillar regurgitant application. Simulated herbivory in v1 and v3 plants elicits jasmonic acid production, indicating that v1 does in fact respond to caterpillar regurgitant. We hypothesized that v1 plants may rely on direct instead of

indirect defenses. Here, we show that v1 caterpillar-fed plants contain higher transcript levels of a cystatin proteinase inhibitor. Bioassays were conducted to assess larval performance on the two stages. Total larval mass was reduced on v1 compared to v3 plants. These results suggest that expression of plant defenses may shift in their expression throughout a plant's life.

Materials and Methods

Plants, Insects, and Caterpillar Regurgitant Collection

Maize seeds (*Zea mays* hybrid "Bonus", Territorial Seed Co., Cottage Grove, OR) were treated with Flint WG fungicide (Bayer CropScience, Research Triangle Park, NC, USA) and planted in autoclaved soil (Sun Gro Metromix 200, Alberta, CAN) to germinate at 25°C, 12:12 light cycle. Upon emergence, the plants were removed from the soil, the roots were washed thoroughly with water, and transferred to plastic cups (0.5 L) containing a hydroponic growing solution (0.3 L). A modified Ruakura solution [279,280] containing: 2 mM KNO_3, 1 mM NH_4NO_3, 1 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.75 mM $MgSO_4{\cdot}H_2O$, 0.5 mM KH_2PO_4, 0.25 mM NaCl, 0.25 mM K_2SO_4, 0.1 mM FeNaEDTA, 50 µM H_3BO_3, 15 µM $MnCl_2{\cdot}4H_2O$, 2 µM $ZnSO_4{\cdot}7H_2O$, 0.25 µM $CuSO_4{\cdot}5H_2O$, and 0.2 µM $Na_2MoO_4{\cdot}2H_2O$, was prepared in distilled water.

Beet armyworm (BAW), *Spodoptera exigua,* eggs (Benzon Research, Carlisle, PA) were transferred to artificial diet (Southland Products Inc., Lake Village, AR) and reared at 27°C. BAW eggs were stored for two days in the refrigerator before transferring to artificial diet at 27°C. Before feeding experiments or regurgitant collection, the larvae were transferred to individual diet cups containing cut maize tissue and allowed to feed for at least 16 hours. For ease of collection and to prevent contamination, fourth instar larvae were used for regurgitant collections. The collection apparatus consisted of a vial (4 mL) with a screw cap fitted with a

Teflon septum. A microcapillary tube (10 µl-20µl, VWR, West Chester, PA) was inserted into the vial thru the septum along with a second port connected to a vacuum source. Regurgitant was collected into this apparatus with the vial cooled in a dry ice/acetone bath. The regurgitant was boiled to prevent degradation of fatty acid amino conjugates and stored at-80°C [281].

Simulated and actual herbivory treatments

At the v1 stage (one visible leaf collar, three leaves, ten days from seeding) or the v3 stage (three visible leaf collars, five leaves, fourteen days from seeding) (Fig. 1), plants were treated either with beet armyworm *Spodoptera exigua* regurgitant or caterpillars in the afternoon at the 8^{th} or 6^{th} hour of the light cycle. Mechanical damage was performed using a mechanical wounding tool resembling a small pair of wooden tongs. On one tong, an aluminum grater, similar to a nutmeg grinder or a pedicure callus remover, is attached. A Teflon septum is affixed to the other tong. A metal screw near the opening permits adjustment of the wound intensity. For volatile collections, three leaves were wounded. Each leaf had two wound sites in parallel to the midrib. Each wound site received either water (5 µl) or boiled beet armyworm regurgitant (5 µl). For JA analysis, the second youngest leaf was wounded. For larval infestations, three second to third instar larvae were added to the whorl immediately prior to volatile collections which, began in the afternoon and continued for the next twenty two hours into the next morning. Samples for volatile collections were taken from time of treatment, at two or three hour intervals during the light cycle, excluding the dark cycle. Larvae were permitted to feed throughout the collection period. Material for transcript quantification was collected at the end of the experiment.

Intact bioassay

Six neonates were transferred with a small paintbrush to the whorl of each maize plant. The plants and caterpillars were enclosed in an air-permeable bag (13 in width by 24 in length, perf-o-film, Europack Inc., Hockessin, DE, USA) that was secured with a rubber band. Plants were watered as needed with a diluted hydroponics solution (1:1 v/v water: solution). Plant stage was recorded on a daily basis. After seven days, larvae were removed and weighed. Aboveground plant tissue was weighed. A rating system based on Davis et al. was used to score damage on a per leaf basis [282,283]. Ratings of 10, 8, 6, 4, 2 and 0 corresponded to the following, respectively: 80-100% leaf tissue removed with only leaf veins and midrib remaining, 60-80% leaf tissue removed with some tissue remaining but mostly leaf veins, 40-60% leaf tissue removed with lesions greater than 2.5 cm in length, 20-40% leaf tissue removed with lesions less than 2.5 cm in length, 1-20% leaf tissue removed with only small pinpricks and holes, and no damage visible. Damage ratings were averaged for the 3^{rd}, 4^{th}, and 5^{th} leaves. These leaves were chosen because they had fully expanded and experienced the majority of caterpillar feeding.

Volatile collection and analysis

Volatiles were collected by enclosing the plant in a chamber, pushing purified air into the chamber and over the plant, and pulling the air through an adsorbent to trap the volatile organic compounds. Volatile collection chambers consisted of a modified Pyrex bottle (7 L) with a Teflon stopper with an inlet for charcoal filtered air (1.5 L min^{-1}) and an aluminum guillotine at the bottom of the chamber [284]. The plant remained in its growing container and the stem was wrapped in cotton with the guillotine surrounding the stem. Fabricated filter traps were attached to vacuum (1.0 L min^{-1}) via ports at the bottom of the chamber. The filter traps were constructed

as described previously [284]. Each filter trap contained an adsorbent (30 mg, Super Q, 80/100 mesh, Altech Associates Inc., State College, PA).

To analyze the emitted volatiles, filter traps were eluted (100 µl, 1:1 v/v hexanes (J.T. Baker): dichloromethane (Honeywell)) and internal standard was added (20 µl, 7.5 ng μl^{-1} octane, 8.5 ng μl^{-1} nonyl acetate). Samples (1 µl injection volume, splitless) were analyzed with a gas chromatograph-flame ionization detector (Agilent 6890N) fitted with an Equity-1 column (Agilent, Santa Clara, CA, USA, 15 m x 0.25 mm ID x 0.25 µm film thickness) using helium as the carrier gas at an average linear flow velocity of 24 cm per second. The oven program was 40°C, 1 min; 8°C/min to 180°C; followed by a program of 30°C/min to 300°C, and held for 5 min.

Data were acquired and peak areas integrated with Agilent MSDChemStation software. Identified compounds were quantified by comparing the peak areas of the compounds of interest with the peak area of the internal standard, nonyl acetate. Compound identity was verified by comparing mass spectra and retention times of collected compounds with standards. In addition, spectra were compared to the National Institute of Standards and Technology (NIST) mass spectral library (2002). For analysis, the gas chromatograph-mass spectrometer (GC-MS), oven program and injection parameters were the same as above. The GC-MS was fitted with a HP-1MS column (Agilent, Santa Clara, CA, USA, 30 m x 0.25 ID x 0.25 µm) with helium as the carrier gas at an average linear flow velocity of 30 cm per second. In addition, spectra were compared to the National Institute of Standards and Technology (NIST) mass spectral library (2002).

Total volatile amounts were calculated by summing the quantities of green leaf volatiles ((*E)*-2-hexenal, (*Z)*-3-hexen-1-ol, (*Z)*-3-hexenyl acetate), monoterpenes (α-pinene, β-pinene, β-myrcene, α-phellandrene, α-terpinene, limonene, ocimene, linalool, geranyl acetate), homoterpenes (4,8-dimethyl-1,3,7-nonatriene, 4,8,12-trimethyl-1,3,7,11-tridecatetraene),

aromatics (indole, phenyl ethyl acetate), and sesquiterpenes (β-caryophyllene, *(E)*-β-farnesene, bergamotene).

Jasmonic acid extraction and analysis

JA analysis was performed using the vapor phase extraction method [285]. The procedure involves five basic steps: tissue homogenization, preparation of the organic phase, derivatization, vaporization, and elution. Briefly, 20 mg-100mg of maize tissue was harvested into pre-weighed tubes containing Zirmil beads (~0.7 g, 0.9-1.1mm diameter, Saint-Gobain, Le Pontet, FRA), solvent (400 µl, 2:1 acidified isopropanol: water, pH 3), and an internal standard mix (20 µl, 10 ng per µl) and immediately frozen in liquid nitrogen. The internal standard mix consists of dihydrojasmonic acid, indole-2,4,5,6,7-d5-3-acetic acid (C/D/N isotopes), 2-hydroxybenzoic acid d6 (C/D/N isotopes), (±)-2-cis,4-trans- abscisic acid-d6 (Icon isotopes), and gamma linolenate (Sigma).

Prior to the extraction, the frozen samples were placed on ice and quickly re-weighed to determine the tissue mass. Each tube was wiped with a kim-wipe to remove most of the frost. Method blanks were included in each analysis to account for differences in weight due to any residual frost and condensation.

Samples were homogenized (40 s, FastPrep, ThermoSavant) and cooled on ice. Dichloromethane (1 mL) was added, followed by another homogenization (20s), and cooled on ice. The pH of the aqueous layer was adjusted to three using hydrochloric acid (2N). The organic layer was removed and evaporated under a stream of nitrogen.

Samples were methylated by introducing trimethyl silyl diazomethane (3 µl, 2M in hexane) and ether:methanol (200 µl, 9:1 v/v) with a gentle vortexing to thoroughly mix contents.

The reaction continued for thirty minutes. The contents of the vial were evaporated under a stream of nitrogen.

A filter trap was attached to a vacuum line (0.5 L min^{-1}) and inserted into the vial. Samples were vaporized and collected onto filters containing an adsorbent (30 mg, Super Q, 80/100 mesh, Altech Associates Inc., State College, PA) at 180 °C for two minutes. The filters were eluted with dichloromethane (150 µl).

Samples (1 µl injection volume, splitless) were analyzed with a gas chromatograph- mass spectrometer (Agilent) in chemical ionization mode using selected ion monitoring and fitted with a HP-1MS column (Agilent, 30m x 0.25 mm ID x 0.25 µm film thickness) with helium as carrier gas at an average linear flow velocity of 30 cm per second. The ionization gas was isobutane. The oven program was 40°C, 1min; 15°C per min to 300°C, and held at 300°C for 5 min.

Primer design

Proteinase inhibitor primers were designed in collaboration with Dr. Irmgard Seidl-Adams (Table 1). TPS10 and FppS3 primers were designed and verified by Dr. Irmgard Seidl-Adams. Primer specificity was verified by sequencing PCR products.

RNA extraction, DNase treatment, cDNA synthesis, qPCR

The tissue was collected at the end of the volatile collection, as described above, and immediately frozen in liquid nitrogen. Samples were stored at -80 °C until extraction. RNA was extracted from caterpillar-damaged maize whorl tissue, approximately 20-100 mg, with the RNeasy Plant Mini kit (Qiagen) and treated with DNase (Turbo DNA-free kit, Ambion). RNA amounts were quantified using a spectrophotometer (NanoDrop, Thermo Scientific, Wilmington,

DE). DNase-treated RNA (5 µg, 9.5µl) was reverse transcribed with SMART MMLV reverse transcriptase (Clontech) and diluted (1:10, v/v, cDNA: water) and used in 5 µL aliquots. Quantitative PCR reactions (20 µL) were run in triplicate on a Bio-Rad iQ5 cycler, containing diluted cDNA (5 µL) , primer mix (10 µM each, 1 µL), SsoFast EvaGreen master mix (10 µL, Bio-Rad), and water (4µL). Transcript levels were quantified using a reference gene and a standard curve constructed of five 3-fold serial dilutions of pooled cDNA.

Statistical Analysis

Analyses were performed with Minitab release 16.1 (State College, PA, USA). Data were transformed as needed to satisfy normality. Two-way ANOVA was conducted using the General Linear Model command of Minitab.

Results

Volatile induction by simulated herbivory

V3 plants produced more total volatiles compared to v1 plants (Fig. 2). Caterpillar spit was applied to artificially wounded v1 and v3 stage maize plants in the afternoon. Volatiles were collected from time of treatment into the next light cycle at two to three hour intervals, excluding the dark cycle. The volatile data were normalized using a mean fresh weight for each stage (mean ± SE g: v1 1.6 ± 0.06, v3 6.8 ± 0.2). Induction of total volatiles failed to occur in spit treated v1 plants. This led to the question of whether v1 plants actually respond to simulated or actual herbivory.

Jasmonic acid levels in response to simulated herbivory

To assess v1 plant response to herbivory, jasmonic acid was chosen as an indicator of an active defense response. Typically, the highest induction of jasmonic acid in maize, due to simulated herbivory, occurs thirty minutes after elicitation [71]. Plants of both stages received artificial wounds along with spit application in the afternoon and leaf tissue was harvested for jasmonic acid quantification thirty minutes after treatment. Both, v1 and v3, maize plants contain elevated levels of jasmonic acid post-spit application compared to controls (Fig. 3). The JA levels are not significantly different in the v1 and v3 spit-treated plants. Once we had established that JA indicated a defense response, we sought to determine whether v1 plants responded to actual caterpillar feeding.

Volatile induction by actual herbivory

To determine whether v1 and v3 maize respond to BAW feeding, larvae were placed in the whorl of v1 or v3 stage plants in the afternoon, enclosed in volatile collection chambers, and allowed to feed for the next twenty two hours. The headspace of the maize plants was continuously sampled at two to three hour intervals for the next 22 hours. Volatiles were not collected during the dark cycle. Caterpillar feeding in v3 maize elicits a blend dominated by sesquiterpenes, as previously shown in caterpillar damaged maize [80]. However, sesquiterpene induction in v1 maize is relatively modest compared to indole, a bicyclic aromatic compound, which dominates the volatile profile (Fig. 4). The larger plants, v3, produce more total volatiles when not normalized for fresh weight (Fig. 4). To further characterize the indirect defense response of v1 and v3 plants, we measured transcript levels of terpene biosynthetic enzymes.

Terpene biosynthetic enzyme transcript levels in caterpillar-fed maize

V1 plants, regardless of herbivory treatment, induce very low amounts of sesquiterpenes. This led to the question of whether lower sesquiterpene production was reflected at the transcriptional level. We approached this by measuring transcript levels of a terpene synthase (TPS) and farnesyl pyrophosphate synthase (FppS). FppS, upstream of TPS10, synthesizes farnesyl pyrophosphate which is the substrate of TPS10. TPS10 catalyzes the transformation of farnesyl pyrophosphate into *E*-β-farnesene and *E*-α-bergamotene [269]. We used quantitative real-time PCR to measure transcript levels of TPS10 and FppS3. A single time point was used at the end of the volatile collection in which maize plants had experienced twenty two hours of continuous BAW feeding. Caterpillar feeding elicited an increase in TPS10 and FppS3 transcripts (Fig. 5A,B). The relative abundance of FppS3 was lower in v1 plants although not significantly different ($p = 0.07$). TPS10 expression was higher in v1 maize (Fig. 5B).

Proteinase inhibitor transcript levels in caterpillar-fed maize

In order to further characterize the defense response of seedling maize, we measured transcript levels of three proteinase inhibitors. The candidates were selected from expression data reported for herbivore-fed maize [184]. Two proteinase inhibitors, maize proteinase inhibitor (Fig. 5D) and a serine proteinase inhibitor were induced by caterpillar feeding in both stages with higher transcript levels, although statistically non-significant, in v1 maize. Data is not shown for the serine proteinase inhibitor because the pattern is similar to maize proteinase inhibitor (Fig. 5D). The screening process revealed that the serine proteinase inhibitor had roughly equivalent expression in caterpillar-fed v1 and v3. A cystatin-like proteinase inhibitor had significantly greater transcript levels in v1 compared to v3 caterpillar-fed maize (Fig. 5C).

Larval performance on v1 and v3 maize

Bioassays with intact seedlings were performed to compare the impact of v1 and v3 direct defenses on beet armyworm, *Spodoptera exigua*, larval performance. Bioassay data indicate that total larval mass was reduced when feeding on v1 compared to v3 plants (Fig. 6). Observed plant damage was not consistently different among the experimental replicates.

Discussion

Jasmonic acid indicates an active defense response to simulated herbivory in v1 and v3 maize

Many physiological and defensive functions including anther dehiscence, pollen development, tuberization, senescence, flowering, drought, UV light, wounding, necrotrophic pathogens, and insects elicit or require the presence of JA [173]. In response to insect feeding, elevated JA levels correlate with the induction of direct and indirect defenses. In maize, JA initially peaks at thirty minutes after mechanical damage and the addition of beet armyworm, *S. exigua*, regurgitant [71]. V1 and v3 plants produced similar amounts of JA in response to simulated herbivory (Fig. 3). This suggests that although spit treatment fails to induce volatile production in v1 plants (Fig. 2), the plants are responding.

Indirect defense expression in v1 and v3 maize

Simulated herbivory did not elicit higher total volatile production in v1 compared to v3 maize. Total sesquiterpene amounts were significantly higher in regurgitant-treated than mechanically wounded or control v1 maize seedlings. However, sesquiterpenes were a minor,

induced component of the v1 maize volatile profile, less than 10% of total volatiles. This is in contrast to our findings with regurgitant-treated v3 maize that had a sesquiterpene dominant blend, nearly 60% of total volatiles. This could suggest that v1 maize may not respond to the components present in boiled regurgitant. However, regurgitant-treated v1 plants did increase JA levels. Also, v1 maize seedlings produced volatiles in response to caterpillar feeding, which suggests that v1 seedlings respond to enzymatically active regurgitant components, other oral secretions, or continuous mechanical wounding. Plant response to caterpillar feeding is exceedingly complex with elicitors and suppressors present in the oral secretions [109,118,286].

Caterpillar-damaged v1 and v3 maize produced a similar amount of volatiles when normalized to amount of leaf material. However, the profiles were markedly different. V1 plants produced a predominance of indole and significantly less sesquiterpenes than v3 plants. We investigated the low sesquiterpene induction in v1 caterpillar fed plants by measuring transcript levels of FppS3 and TPS10. Despite our expectations, TPS10 had higher transcript levels in v1 than in v3. However, FppS3 upstream of TPS10 had significantly lower expression in v1 than in v3 plants. This suggests that the regulation of sesquiterpenes in v1 maize may occur at FppS3 or even further upstream. However, transcript levels do not necessarily correlate with protein abundance or activity. Alternatively, reduced sesquiterpene production may result from substrate limitation.

Direct defense expression in v1 and v3 maize

Due to the low amount of volatile production in v1 plants, we hypothesized that v1 plants may allocate more of their resources to direct defenses. To assess direct defense expression, proteinase transcript levels and larval performance were measured in both stages. Many plant species utilize proteinase inhibitors to limit herbivore growth and survival [41,287,288]. PIs are

small proteins, which interfere with the digestion of proteins within the insect gut [185]. In addition, application or elevation of JA is correlated with proteinase inhibitor production [289]. In maize, pathogen infection and insect feeding induce accumulation of maize proteinase inhibitor transcripts [249,251]. From Ton et al. (2007), we selected four proteinase inhibitor genes that were induced by caterpillar feeding in maize [184]. Caterpillar feeding induced the accumulation of all four PIs in v1 and v3 maize (Fig. 5C, D). Larval performance on v1 was reduced compared to v3 maize (Fig. 6). Although transcripts of a cystatin-like proteinase inhibitor were higher in v1 compared to v3 caterpillar damaged plants, cystatin PIs inhibit cysteine proteases, which Lepidoptera do not use for digestion [39]. It is important to note that we evaluated the expression of a very small subset of inducible PIs and did not consider any constitutively expressed PIs. The maize genome contains at least 80 annotated proteinase inhibitors. Thus, we present an experimentally feasible but perhaps, biologically narrow picture of inducible PI transcripts. Additionally, v1 seedlings may have higher amounts of constitutive proteinase inhibitors, which are highly expressed during germination [249,290]. Therefore, reduced larval performance on v1 plants is probably attributable to other direct defenses. For instance, members of the Poaceae, grass family, produce cyclic hydroxamic acids or benzoxazinones, toxic secondary metabolites that can impede larval growth [240]. These compounds change in abundance during seedling development with a trend of decreasing concentration a few days following germination [235]. Not only are benzoxazinones constitutively expressed at certain developmental stages but these compounds can be induced by fungal infection and herbivory [237,238]. Further work needs to establish both constitutive and induced levels of benzoxazinones in herbivore-damaged v1 and v3 maize seedlings. Contrary to the expectation of a very young seedling unable to defend itself, herbivory elicits transcript accumulation of proteinase inhibitors in v1 seedlings. In addition, v1 seedlings effectively reduce larval growth compared to v3 seedlings.

Conclusion

In this study we compared the response of two maize seedling stages to actual and simulated herbivory. Maize volatile induction at the v1 stage is rather limited. Interestingly, mechanical wounding or other factors in the caterpillar's oral secretions may be the stimulus for volatile production in v1 seedlings. Jasmonic acid levels indicated that both stages respond to simulated herbivory. Despite low volatile induction in v1 plants, this stage induces proteinase inhibitor transcript levels to an equal or greater extent than v3 plant. Beet armyworm, *Spodoptera exigua*, larval performance was reduced on v1 compared to v3 maize plants. These data suggest that v1 plants actively respond to herbivory and may have a more inducible direct defense and a subdued indirect defense system. The timing and type of defense expression in maize may have important implications for pest management strategies in agriculture.

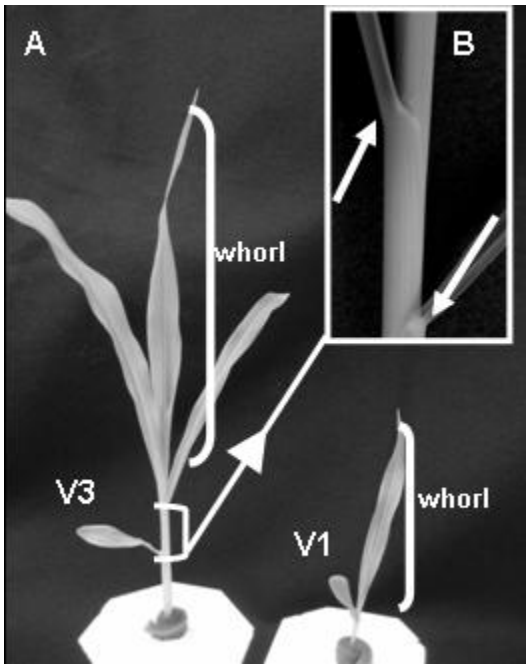

Figure 2-1: Image of V1 and V3 developmental stages. A) V1 and V3 stage plants with whorl portion indicated by brackets. B) Inset with arrows pointing to leaf collars, which are used to determine the developmental stage.

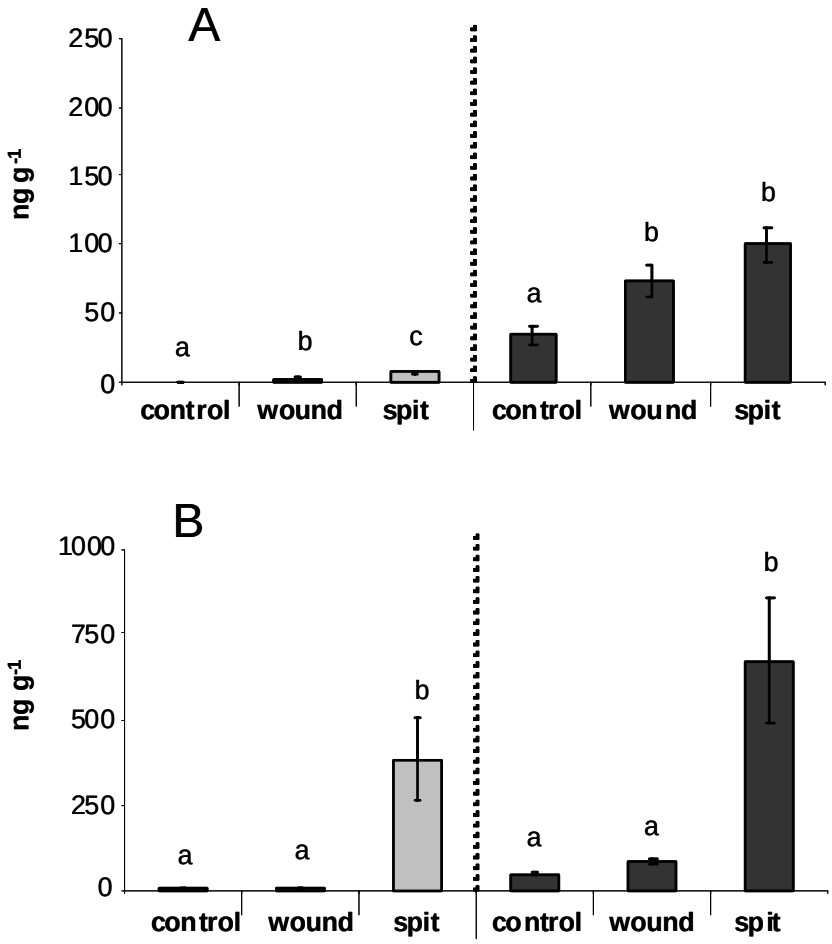

Figure 2-2: Volatile response of v1 and v3 maize to simulated herbivory. Amounts (means ± SE) of ☐ sesquiterpenes, and ■ total volatiles produced by **A)** v1 and **B)** v3 maize in response to mechanical damage and caterpillar regurgitant application (spit), mechanical damage control (wounding), and undamaged (control). This time is at the maximum of volatile production, eighteen hours after the simulated herbivory treatment. These volatiles were collected between the 1st and 3rd hours of the light cycle. Volatile amounts are normalized by amount of tissue. Data represent 4 biological replicates per treatment and 3 and 5 experimental replicates for v1 and v3. The data were log transformed. A two-way ANOVA included a significant interaction of stage and treatment ($F_{2,17}$=15.88, $p<0.0001$). Additionally, treatment ($F_{2,17}$=65.08, $p<0.0001$) and stage ($F_{1,17}$=36.93, $p<0.0001$) effects were significant. One-way

ANOVA was performed for each stage to compare total sesquiterpenes and total volatiles between treatments.

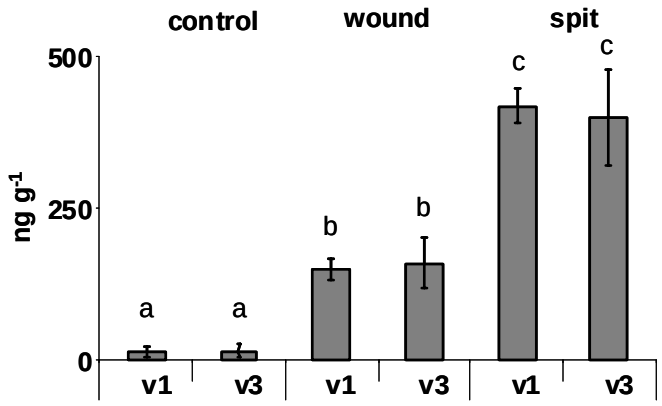

Figure 2-3: Jasmonic acid (JA) levels in v1 and v3 maize after simulated herbivory. JA level (means ± SE) of v1 and v3 maize thirty minutes after simulated herbivory (spit), mechanical damage control (wounding), and undamaged (control). Jasmonic acid amounts are normalized to plant fresh weight (g). Data represent 6 biological replicates per treatment and 3 experimental replicates. The data were square root transformed and compared using the Tukey method.

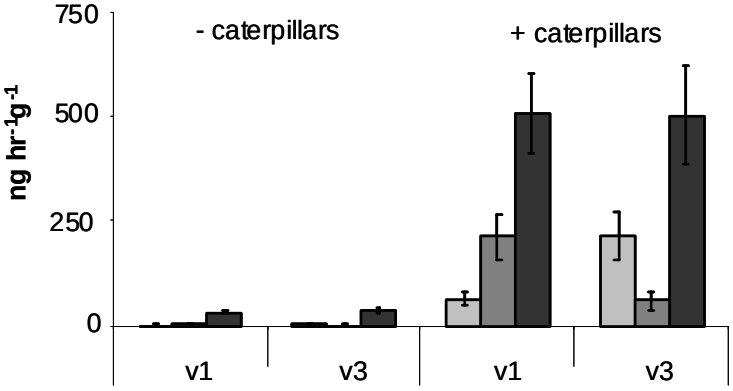

Figure 2-4: Volatile response of v1 and v3 maize to caterpillar feeding. Amounts (means ± SE) of ☐ sesquiterpenes, ☐ aromatics, and ☐ total volatiles produced by v1 and v3 maize in response to 20 hours of continuous caterpillar feeding (+caterpillars). This time is at the maximum of volatile production after twenty hours of caterpillar feeding. These volatiles were collected between the 2nd and 4th hours of the light cycle. Volatile amounts are normalized to fresh weight mass (g). Data represent 8 (+caterpillars) and 4 (-caterpillars) biological replicates. Experiments were repeated three times. The data were log transformed and compared using the Tukey method.

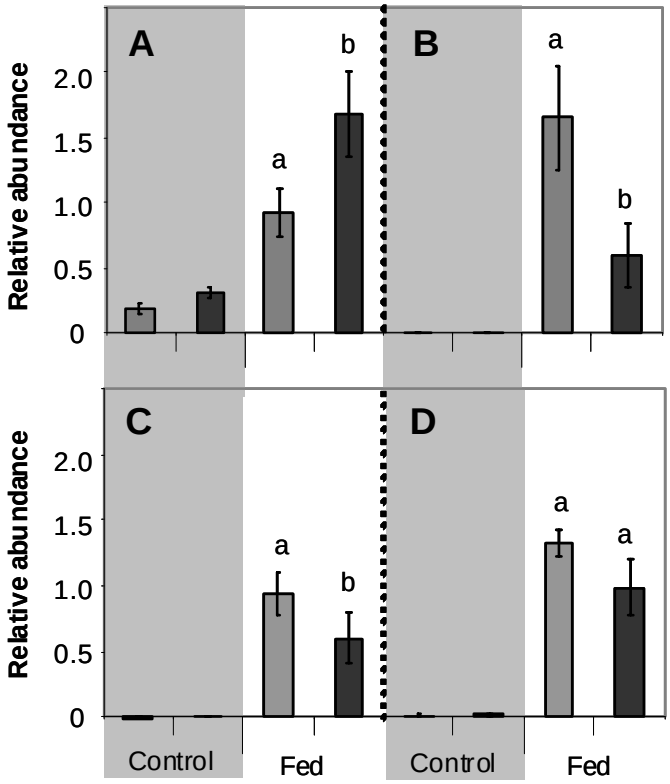

Figure 2-5: Expression of terpene biosynthetic and proteinase inhibitor genes in v1 and v3 maize after caterpillar feeding. Abundance of transcripts (mean ± SE) of (A) farnesyl pyrophosphate synthetase FppS3, (B) terpene synthase TPS10, (C) cystatin-like proteinase inhibitor, and (D) maize proteinase inhibitor from ■ v1 and ■ v3 plants after 22 hours of continuous feeding. Data represent 8 biological replicates. Induction of transcripts was verified by analyzing expression in four undamaged seedlings per stage. Comparison of v1 and v3 caterpillar-fed treatments performed with an unpaired Student's t-test, $\alpha = 0.05$, on square-root transformed data. For A and B, a one-tailed test was performed because based on the volatile data, we hypothesized that transcript levels of terpene biosynthetic enzymes would be reduced in v1 plants. Abundance was calculated using a reference gene.

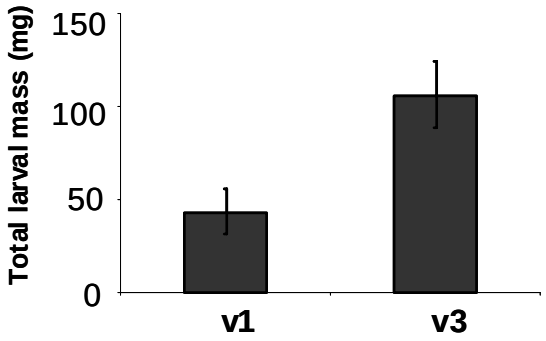

Figure 2-6: Larval performance on v1 and v3 maize. Each plant received six S. exigua neonates. After seven days, the larvae were removed from the plants and weighed. Data represent mean ± SE of ten larval cohorts per developmental stage. Two experimental replicates were performed. Treatments were compared using an unpaired Student's t-test. Larvae masses were significantly different ($t=-2.89$, d.f. 18, $p<0.01$).

Table 2-1: A list of primer sequences used for qPCR analysis.

ID	FORWARD PRIMER (5' TO 3')	REVERSE PRIMER (5' TO 3')	GENBANK ACCESSION NUMBER
Maize PI	ATGAGCTCCACGGAGTGC	TCAGCCGATGTGGGGCGTC	X78988
Cystatin-like PI	CCAGAGTCCATAGACATCCA	TACGGATGATCCAGTGACAG	BM072984
Serine PI	CCGCTTCAGAGAAGAACTAA	AAGTGAAGTGAAGTGACGATG	BM382058
Cystatin-like PI	AGGGCTTGTTCGGTTAGGTG	TGCAGAATAAGGAGCCATGC	CK827737

Chapter 3

Priming in maize negatively effects the herbivore, *Spodoptera exigua,* but lacks typical defense response indicators

Abstract

Primed plants preemptively coordinate their defense system by utilizing environmental cues to induce a state of readiness. Once the primed state is initiated, a primed plant will respond with a faster, stronger defense response, when an herbivore attacks. The primed state was initiated by exposure to a green leaf volatile, (*Z)*-3-hexenyl acetate and verified by measuring jasmonic acid production thirty minutes after exposure. To establish whether primed maize negatively affects the herbivore, we performed bioassays with beet armyworm, *Spodoptera exigua,* neonates using excised plant tissue and intact plants. Larvae feeding on primed excised maize tissue experienced a delay in instar progression but no increase in mortality. Intact plant bioassays were conducted with two seedling maize developmental stages, v1 and v3. Interestingly, primed v1 maize reduced larval weights and experienced less damage. This does not appear to be the case for v3 maize. To assess how the maize plant might reduce larval growth, we characterized the elicited, primed defense response due to caterpillar feeding by measuring JA levels, volatile production, and proteinase inhibitor transcripts. Contrary to other findings, these indicators did not distinguish between caterpillar damaged primed and control maize at either developmental stage. These findings indicate that priming reduces herbivore growth and may be developmentally specific in maize.

Introduction

Roots connect and imprison plants to their local environment. Their sedentary lifestyle prohibits them from re-locating to avoid disease, insect damage, drought, adverse weather, or growth-inhibiting soil conditions. These environmental stresses invoke plants to exhibit intricate and poorly understood response mechanisms. In order to survive and reproduce, plants must respond and either tolerate or defend themselves from an array of stresses. So, how do plants respond to their environment? In this chapter, I address how plants defend themselves against insect attack or herbivory using priming, a defensive tactic that minimizes energetic investments by maximizing defense expression only when a threat is present. To trigger the primed state, an undamaged plant is exposed to a volatile compound or blend, which is typically produced by herbivore-damaged leaf tissue. After initiating the primed state, a primed plant responds to a stress elicitation with stronger, faster defense or tolerance [119]. This phenomenon is not limited to plant-insect interactions but also applies to plant-pathogen and plant-abiotic stress interactions [120]. Nor is it restricted to volatile signals.

Between plant signaling to induce herbivore resistance has been a controversial subject since the first report [180]. Experimental design, location i.e. field or laboratory, and inconsistent findings drew criticism and skepticism regarding the phenomenon [181,182]. Because the mechanism was unknown, a contributing factor to the contradictory results was analysis for herbivore resistance traits before herbivore damage. This is not surprising when the cost of producing defense(s) without the presence of an herbivore can outweigh the benefits. An alternative explanation emerged that perhaps, plants primed their defenses for a faster, stronger response once an herbivore begins to feed. The receiver plant or leaf does not induce a resistance trait at the time of stimulus perception but in fact waits until herbivory begins to initiate a stronger, faster response.

Recent research suggests that priming may explain how undamaged plants react to signals from damaged neighbors. For example, damaged wild sagebrush produces volatiles that signal to adjacent tobacco plants causing the tobacco plants to prime their defenses [183]. Moving down a spatial level from plants to leaves, recent studies in poplar, lima bean and blueberry established that damaged leaves can prime adjacent undamaged leaves [92,171,175]. Priming may confer an advantage to plants in an environment where herbivory is likely but not certain to occur. Although it appears that eliciting a primed plant via simulated herbivory results in a faster and elevated defense response, the defense response due to caterpillars feeding on primed maize and the potential negative impacts on the herbivore are unknown or poorly understood.

Few have addressed whether elicitation of a primed plant negatively affects the feeding herbivore. Tobacco hornworm, *Manduca sexta*, larvae feeding on primed tobacco plants experienced higher mortality [183]. Beet armyworm, *Spodoptera littoralis*, larvae gained less weight on primed maize compared to control maize after eleven hours of feeding but not at later time points [184]. In addition, transcript levels of several proteinase inhibitors were elevated in primed plants 500 minutes after elicitation with caterpillar regurgitant. Typically, plants contain higher transcript levels or inhibitor activity during herbivory and these are associated with reduced caterpillar performance and survival [184,185,186]. So, how does feeding on a primed maize plant affect an herbivore?

In defense against herbivory, plants can continuously express constitutive defenses and/or induce defenses once a threat is present. Plants express defenses using these two modes. In many cases, plants constitutively produce low or moderate levels of a toxic metabolite, or xenobiotic, but will induce to greater amounts when herbivore damage occurs. For instance, parsnip leaves, *Pastinaca sativa*, maintain microgram quantities of furanocoumarins that double after cabbage looper feeding [9]. However, the parsnip webworm, a specialist herbivore, prefers

to feed on the fruits Because of high probability of attack and value for reproductive fitness, the parsnip fruits contain high constitutive levels of furanocoumarins that are not increased by artificial damage [10]. Ultimately, defense expression is context and cost dependent.

Both constitutively and inductively regulated defenses can function either directly or indirectly defend the plant. During caterpillar feeding, plants induce direct and indirect defenses to reduce or deter feeding [5]. Plant defenses may directly repress herbivore growth and survival by modifying structural and chemical leaf traits. In addition, a plant may indirectly defend itself through the production of volatiles or extrafloral nectaries to attract predators and parasitoids of the herbivore [80,291]. The defense response to herbivory has been well-characterized across plant species and insect feeding habits [6,8]. However, at a mechanistic level, it remains largely unknown how defenses are induced.

Understanding of how eliciting a primed plant affects the herbivore will provide context for investigating the defense response of primed plants. The practical applicability is enhanced by characterizing this phenomenon in a plant of agronomic importance. The majority of interplant signaling and priming studies have examined woody perennial tree species and wild herbaceous annuals but not cultivated species. One of the first studies to demonstrate that a volatile signal can prime a plant used a hybrid dent corn hybrid 'Delprim' [71]. Previous studies established that primed maize plants contain elevated levels of JA, proteinase inhibitor transcripts, and volatile production after simulated herbivory [71,184]. Maize is an ideal study organism for interplant priming for two reasons. Caterpillar feeding tends to occur in the whorl, the site of developing leaves, which localizes the emitted volatile organic compounds. Also, maize, either in small subsistence plots or vast agribusiness fields, is grown with minimal spacing that maximizes the likelihood that volatiles will travel between plants [160].

Here we investigate whether primed maize negatively affects caterpillar growth and how a primed plant might suppress this growth. Previous research suggested that v1 maize contained

equivalent or higher transcript amounts of several proteinase inhibitors (see Chapter two). We hypothesized that primed v1 maize may limit larval growth to a greater extent than primed v3 maize. We verified that four maize genotypes and two developmental stages responded to exposure to (*Z*)-3-hexenyl acetate, the priming agent. The two developmental stages were both seedling stages that had either one or three fully developed leaf collars, v1 and v3, which roughly corresponds to three or five leaves. Initial experiments with excised primed tissue indicated that primed maize delays instar progression. Our subsequent approach incorporated a more realistic bioassay design with intact plants and a comparison of two vegetative stages of maize. Our results suggest that priming in maize reduces larval performance and that this effect differs by maize developmental stage. However, transcript levels of four proteinase inhibitors were not higher in induced, primed maize. Caterpillar feeding induced volatile production in both maize developmental stages but was not enhanced in primed plants. Thus, although larval performance was inhibited, our results did not show any indication of an elicited primed defense response in plants damaged by caterpillar feeding.

Materials and methods

Plant and Insect Rearing

Plants were grown in either hydroponics or a commercial potting mix. To prevent infection from seed-borne fungi, maize seeds (Inbred lines: B73, Tx601, and W438) were treated with Flint WG fungicide (Bayer CropScience, Research Triangle Park, NC, USA). Seeds, of B73 and W438, were germinated in autoclaved soil (Sun Gro Metromix 200, Alberta, CAN) at 25°C, 12:12 light cycle. Upon emergence, the plants were removed from the soil, the roots were washed thoroughly with water, and transferred to plastic cups (0.5 L) containing a hydroponic

growing solution (0.3 L). Plants were used at the v1 stage. This stage corresponds to a plant roughly 10 days from seeding that bears three leaves with one fully developed leaf collar (Fig. 1). A modified Ruakura solution containing: 2 mM KNO_3, 1 mM NH_4NO_3, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.75 mM $MgSO_4 \cdot H_2O$, 0.5 mM KH_2PO_4, 0.25 mM NaCl, 0.25 mM K_2SO_4, 0.1 mM FeNaEDTA, 50 μM H_3BO_3, 15 μM $MnCl_2 \cdot 4H_2O$, 2 μM $ZnSO_4 \cdot 7H_2O$, 0.25 μM $CuSO_4 \cdot 5H_2O$, and 0.2 μM $Na_2MoO_4 \cdot 2H_2O$, was prepared in distilled water [279,280]. Tx601 maize plants were grown in autoclaved soil at 25°C, 16h light:8h dark cycle, until the v1 or v3 (5 leaves, 3 fully developed leaf collars, 14 days from seeding) (Fig. 1). At seeding, a slow release fertilizer was added to each pot (~2g, Osmocote Plus, 15-9-12, Scotts-Sierra Horticultural Products Co., Marysville, OH, USA).

Beet armyworm (BAW), *Spodoptera exigua* , eggs (Benzon Research, Carlisle, PA) were transferred to artificial diet (Southland Products Inc., Lake Village, AR) and reared at 27°C. For excised bioassays, the eggs were stored in the refrigerator for seven days. In the intact bioassay, the eggs were used when received and only experienced a few hours of cold storage.

Assessment of primed state after GLV exposure

To verify that maize plants responded to GLV treatment, jasmonic acid levels were measured using the vapor phase extraction method described in Chapter 2. In these experiments, three genotypes and two developmental stages of maize were exposed to a GLV, (*Z*)-3-hexenyl acetate, for thirty minutes.

To prime the plants, they were placed in Plexiglas chambers (7 L total volume) as described by Engelberth et al. [71]. Each chamber received a cotton ball containing (*Z*)-3-hexenyl acetate (20 μg, 1 μg μl^{-1}, dissolved in dichloromethane) or dichloromethane (20 μl). In addition, two other treatments were included in which plants were not enclosed in chambers to

control for any effects from the chamber and another group in chambers with a cotton ball to control for exposure to the cotton ball. After 30 minutes of exposure, each maize plant was removed from the chamber and approximately 100 mg of the youngest leaf was harvested and frozen immediately in liquid nitrogen.

Excised bioassay

To initiate the primed state, the plants were exposed using the same set-up described above except the exposure period was overnight. The chambers were removed the following morning and the plants were allowed to equilibriate in the growth chamber for at least thirty minutes.

This assay was used to allow for careful monitoring of individual instar progression. Tx601 plants at the v3 stage were used. Plants were primed as described. Beet armyworm (BAW), *Spodoptera exigua*, eggs were stored in the refrigerator for 7 days. Preliminary data indicated that larvae that experienced 7 days of egg cold storage stress, feeding on an insect-susceptible inbred line, B73, or an insect-resistant inbred line Tx601, had enhanced susceptibility to maize resistance traits (Fig. 2). For excised plant bioassays, eggs were stored in a refrigerator at 20°C for seven days. Eggs were hatched on artificial diet at 27°C. Five neonate larvae were transferred to a diet cup containing a thin layer of 1% agar and 100-200 mg of leaf tissue. On day 3, after three days of feeding, larvae were counted to determine neonate mortality rates. The most robust, largest larva was selected for the remainder of the bioassay. Larval instars and weights were recorded on days 3, 4, 6, and 7.

Intact bioassay

V1 and v3 stage, Tx601, plants were primed as described. Ten neonates were transferred with a small paintbrush to the whorl of each maize plant. The plants and caterpillars were encased in an air-permeable bag that was secured with a rubber band. Plants were watered as needed. Plant stage was recorded on a daily basis. After seven days, larvae were removed and weighed. Aboveground plant tissue was weighed. Quantitative leaf damage analysis was not possible for most of the plants due to the amount of leaf tissue removed. Instead, a rating system based on Davis et al. was used to score damage on a per leaf basis [282,283]. Ratings of 10, 8, 6, 4, 2 and 0 corresponded to the following, respectively: 80-100% leaf tissue removed with only leaf veins and midrib remaining, 60-80% leaf tissue removed with some tissue remaining but mostly leaf veins, 40-60% leaf tissue removed with lesions greater than 2.5 cm in length, 20-40% leaf tissue removed with lesions less than 2.5 cm in length, 1-20% leaf tissue removed with only small pinpricks and holes, and no damage visible. Damage ratings were averaged for the 3rd, 4th, and 5th leaves. These leaves were chosen because they had fully expanded and experienced the majority of caterpillar feeding.

Elicitation of primed plants

Tx601 plants were grown as described above. Before feeding experiments, the larvae were transferred to individual diet cups containing cut maize tissue and allowed to feed for at least 16 hours. One hour prior to infestation, the larvae were removed from the cups and transferred to empty cups without food. Third instar larvae instead of neonates were used to ensure adequate feeding for induction of JA and volatiles.

To initiate the primed state, the plants were treated as described above. To compare the responses of primed and control plants to herbivore damage, three early 3rd instar larvae were added to the whorl of each plant. For determining jasmonic acid levels, larvae were removed after 10, 30, or 240 minutes. Damaged leaf tissue (~100 mg, 2.54 cm in length) of the developing leaf was harvested and frozen immediately in liquid nitrogen. For transcript analyses, larvae were added to the whorl of each plant and allowed to feed for a half, six, twenty four, forty eight, and seventy two hours. Plants and larvae were enclosed in an air-permeable bag and secured with a rubber band. During feeding time course, the plants were watered in small individual trays from below, as needed. For thirty minutes of feeding, timing was started for each plant when larvae started feeding. To measure volatile induction, plants were primed as described with the following exception: the chamber was a modified Pyrex bottle (7 L) receiving a gentle air flow of 0.3 L min^{-1}. Larvae were added at the onset of the first collection. During the volatile collection (see later), plants were watered in small individual trays from below, as needed.

Jasmonic acid extraction and analysis

JA analysis was performed using the vapor phase extraction method [285]. See Chapter two for details of the procedure.

Volatile collection and analysis

See Chapter two for details of the procedure.

Total volatiles were calculated by summing the quantities of green leaf volatiles ((*E)*-2-hexenal, (*Z)*-3-hexen-1-ol, (*Z)*-3-hexenyl acetate), monoterpenes (α-pinene, β-pinene, β-myrcene, α-phellandrene, α-terpinene, limonene, ocimene, linalool, geranyl acetate), homoterpenes (4,8-

dimethyl-1,3,7-nonatriene, 4,8,12-trimethyl-1,3,7,11-tridecatetraene), aromatics (indole, phenyl ethyl acetate), and sesquiterpenes (β-caryophyllene, *(E)*-β-farnesene, bergamotene).

Primer design

Proteinase inhibitor primers, see Chapter two, were designed using Primer3 and VectorNTI software. Primer specificity was verified by sequencing PCR products and visualizing on an agarose gel. Primer design and subsequent qPCR analyses were done in collaboration with Dr. Irmgard Seidl-Adams.

RNA extraction, DNase treatment, cDNA synthesis, qPCR

The tissue was collected at five time points,as described above, and immediately frozen in liquid nitrogen. Samples were stored at -80 °C until extraction. See Chapter two for details of the procedure.

Statistical Analysis

Minitab version 16.1 (State College, PA) was used for all analyses. The General Linear Model function was used for two-way ANOVA.

Results

JA levels in v1 and v3 maize in response to GLV exposure

To initiate the primed state, maize plants were exposed to (*Z*)-3-hexenyl acetate, a green leafy volatile (GLV). To verify that both v1 and v3 (Fig. 1) developmental stages and an array of genotypes responded to GLV treatment, JA levels were measured after thirty minutes of exposure (Fig. 3, 4). Plants were enclosed in a chamber and primed by adding the GLV or the solvent to a cotton ball in the chamber. To exclude effects from exposure to the cotton ball or enclosure, these controls were included (Fig. 3). After thirty minutes of GLV exposure, leaf tissue from the youngest leaf was harvested to determine the amount of JA. *Cis*-JA levels increased in both stages of primed plants, inbred line Tx601 (Fig. 3) (mean ± SE ng g^{-1}, v1, 107 ± 5; v3, 71 ± 22). To determine whether the JA burst was consistent across genotypes, we repeated the experiment with v1 plants of two inbred lines, B73 and W438. Both inbred lines contained higher amounts of *cis*-JA after GLV exposure (mean ± SE ng g^{-1}, B73, 104 ± 2; W438, 151 ± 18). This suggests that a transient JA burst is a reliable indicator of GLV exposure for this study's two developmental stages and three maize genotypes.

Larval performance on excised v3 maize

Beet armyworm, *Spodoptera exigua*, larvae feeding on primed v3 Tx601 maize experienced a delay in instar progression compared to larvae fed on control maize tissue. Larvae spent a longer amount of time in the second instar when feeding on excised primed maize tissue. After 3 days of feeding, control larvae had all entered the second instar with over half about to molt into the next instar. In contrast, 75% of primed-fed larvae remained in first or second instars with only 25% entering into the second pre—molt stage. Larvae in the primed group spent more

time in the second instar (Fig. 5). Larval weights were not different between control and primed treatments for two maize genotypes (Fig. 6). Mortality can be higher for larvae feeding on primed maize but was not consistent over three replicate experiments. Although, the excised bioassay design limited the scope, it did provide evidence of a negative impact on *S. exigua* larvae. To address limitations of the excised bioassay, bioassays using intact plants were conducted. It was thought that intact plants would more accurately express a primed, elicited defense response.

Larval performance on intact primed v1 and v3 maize

The total mass of larvae that fed on intact primed v1 maize plants for seven days was reduced compared to those fed on v1 controls. Comparing developmental stages, larvae fed on v1 compared to v3 maize had a lower mass (Fig. 7). However, v3 primed plants did not significantly reduce larval mass. Larval survival did not consistently differ between primed and control treatments for both developmental stages (Table 2).

Assessment of primed maize damage and growth during caterpillar feeding

In tune with larval performance, caterpillar damage on v1 primed plants was significantly different from v1 control plants (Figs. 8, 9). Caterpillar damage was evaluated using a modified visual rating scale [282,283]. A visual rating system was used because after seven days of feeding by five to nine larvae, the damage was extensive. For example, the whorl leaves of most v3 plants experienced 80-100% tissue removal with only leaf veins remaining. Quantitative leaf area measurements would have been inaccurate because, in many cases, it was difficult to reconstruct the leaf shape. V3 control and primed plants experienced similar amounts of damage.

At the end of the bioassay, the vegetative portion of the plant including leaf and stem tissues were weighed. Throughout the bioassay, the stage of each plant was recorded to measure the speed of developmental stage progression. No consistent differences were found for vegetative fresh weight (Table 3) or stage progression (Table 4).

Jasmonic acid levels after caterpillar feeding on primed maize

In this study, we wanted to determine whether JA indicated a primed defense response during caterpillar feeding. Maize plants were exposed to a GLV overnight. After GLV exposure, beet armyworm larvae were introduced into the whorl of each plant and allowed to feed for 10, 30, or 240 minutes. Induction of JA by caterpillar feeding was verified by quantifying unfed JA levels in control and primed plants. Caterpillar fed primed and control v3 maize plants did not contain significantly different amounts of JA for all analyzed time points (Fig. 10). Observed damage levels were not different between primed and control plants.

Volatile induction by caterpillar feeding in primed v1 and v3 maize

Primed v1 and v3 maize plants do not produce more volatiles compared to control plants during caterpillar feeding. Volatile organic compounds were collected from initiation of feeding until the next light cycle. Caterpillars feeding on primed and control v1 and v3 plants induced similar amounts of total sesquiterpenes at the time of maximal production, 20-23 hours of feeding, (mean ± SE ng g^{-1}, v1 control fed, 370 ± 103; v1 primed fed, 314 ± 40; v3 control fed, 433 ± 48; v3 primed fed, 433 ± 29). Total volatile production did not differ between developmental stages or priming conditions (Fig. 11). This is consistent with previous results with v1 and v3 ‘Bonus’ maize plants. In Chapter 2, total volatile production when normalized for

plant fresh weight did not differ between caterpillar-damaged v1 and v3 'Bonus' maize plants (Ch. 2, Fig. 4). Although, volatile induction did not differ between control and primed plants; this did not exclude differential expression of direct defenses. Since bioassay data suggests that larval growth is suppressed in primed plants, we measured transcript accumulation of several proteinase inhibitors in primed and control plants during caterpillar feeding.

Time course of proteinase inhibitor transcript accumulation in primed v1 and v3 maize

Bioassay results indicated that a delay in instar progression occurs after only seventy two hours of feeding. Therefore, we postulated that proteinase inhibitor transcripts levels would be elevated in primed fed plants during this period. However, caterpillar feeding over seventy two hours did not induce higher transcript accumulation of proteinase inhibitors in primed v1 and v3 maize compared to damaged control plants. We conducted the experiments using a developmental comparison of v1 and v3 because previous results indicated that primed v1 but not v3 reduced larval weights. A screen of pooled cDNA revealed that transcript levels of four proteinase inhibitors were not elevated in caterpillar fed primed v1 maize compared to caterpillar fed control plants at six, twenty four, forty eight, and seventy two hours. To verify that the time course did not miss an earlier induction, we measured transcript levels after thirty minutes of feeding. Larval feeding induced cystatin-like proteinase inhibitor transcript levels after thirty minutes (Fig. 12). However, transcript levels were not higher in primed caterpillar fed maize.

Discussion

A transient JA burst indicates a response to GLV exposure

To initiate the primed state, a plant is exposed to a biogenic compound such as herbivore induced plant volatiles or to pathogenic and non- pathogenic microbes [119]. Previously, 'Delprim' and 'Bonus' maize plants were shown to contain elevated levels of jasmonic acid shortly after exposure to green leaf volatiles including *(Z)*-3-hexenyl acetate [71,154]. The dose of *(Z)*-3-hexenyl acetate used in this study exceeds the typical level produced by a caterpillar-infested plant. However, JA amounts after exposure to either cut leaf tissue or the current GLV dose were similar in three maize genotypes (Table 1). As previously shown, maize plants exposed to volatiles from caterpillar-infested plants yielded similar amounts of jasmonic acid compared to treatment with GLV compounds [71]. This JA burst was used to confirm that plants entered the primed state. We observed increased JA production after thirty minutes of GLV exposure in two developmental stages and three genotypes of maize (Figs. 3, 4, Table 1). This suggests that JA reliably indicates GLV exposure regardless of maize developmental stage and genotype.

Priming in maize negatively affects larval development

Larvae feeding on intact primed v1 maize had lower weights and experienced less damage. Contrary to the report of Ton et al., our data demonstrated an effect of priming on the herbivore for greater than eleven hours [184]. Although examples of elevated defense responses abound for within and between primed plants, only a few studies have addressed whether a primed plant has any effect upon the herbivore [183,184,190]. It is important to note that these bioassays evaluate direct defense responses and not indirect defenses.

Bioassay data indicated that larvae feeding on excised primed maize tissue experience a delay in instar progression. This may perpetuate into an increased susceptibility of larvae to environmental stresses, parasitization, and predation. Parasitization of *S. exigua* by the wasp, *Meteorus pulchricornis*, was highest in 2nd, 3rd, and 4th instars [292]. In predation assays, 1st and 3rd instar tobacco hornworm, *Manduca sexta,* larvae experienced higher predation compared to 4th instar larvae [293]. Typically, a delay in larval development is associated with lower larval weights or relative growth rates [294,295]. However, the excised bioassay limits the ability to assay for an elicited, primed defense response. Once the tissue is removed from the plant and enters the bioassay set-up, the tissue may not be able to induce primed defenses comparable to that of an intact plant. Therefore, the effect is relatively modest compared to an intact bioassay design and may not reduce larval weights. Prolonging instar duration, especially in early instars, may have a dramatic effect upon herbivore growth and survival.

Caterpillars with a diet of primed excised maize tissue, compared to control tissue, did not appear to differ in individual larval weights. Excised bioassays were conducted to permit observations and measurements of individual larvae to evaluate life history traits including mortality, instar progression, and larval weight gain. Preliminary bioassay data suggested that only "stressed" larvae from eggs that had been in cold storage for five to seven days were negatively affected by primed maize tissue. Cold storage significantly reduced larval weights in two maize genotypes (Fig. 2). Neonates for the excised but not intact bioassays were hatched from "stressed" eggs. However, intact v1 primed compared to control plants did suppress larval growth when "un-stressed" neonates were used.

Despite its value, this study did not measure plant reproductive success and fitness of subsequent generations. This may prove to be the most interesting and long-lasting effect of priming. In wild radish, progeny from herbivore-damaged plants reduced larval weights of a specialist caterpillar [296]. It is not inconceivable that priming may be a maternally inherited

strategy. On the other side of the interaction, herbivore fecundity and resulting larval susceptibility to primed plants needs to be characterized.

Priming in maize may be developmentally specific

Priming and defense utilization may not be expressed equivalently in all plant developmental stages. For instance, proteinase inhibitors, which can reduce herbivore performance, decrease in tomato as it ages [211]. Maize seedlings may have little ability to tolerate herbivore damage since reproductive stages are initiated in the fifth stage [224]. Therefore, reproductive fitness may be negatively affected by loss in the early vegetative stages. Previous research indicated that v1 maize may be more directly defended than v3 maize (see Chapter two). This provided a unique opportunity to compare elicited, primed direct defenses in two developmental stages using larval feeding bioassays. Bioassay results indicate that v1 but not v3 maize engages in an effective primed, direct defense response by reducing larval growth and leaf damage.

However, the herbivore infestations used in the intact bioassay were high and the results may not be the same at lower herbivore infestations. Previous research, in the 'Bonus' hybrid maize, suggested that v1 represses larval growth to a greater extent than v3 plants (mean ± SE mg total larval mass; v1, 44 ± 12; v3 107 ± 18). Indeed, in this study, Tx601 v1 control plants supported less larval growth compared to v3 control plants (Fig. 7). This suggests that perhaps, the v1 stage is more resistant to herbivory. Perhaps, v1 maize contains higher constitutive and induced direct defenses compared to v3. The most obvious explanation would be the correlation of decreasing DIMBOA concentrations as seedlings age [230]. Although rather simplistic, this is a logical starting point for subsequent research. It would be interesting to determine constitutive and induced levels of DIMBOA between developmental stages and priming states.

Aside from resistance, perhaps, priming influences a plant's ability to tolerate herbivore damage. Measures of enhanced tolerance include greater biomass accumulation, maintenance or increase of seed yield, and increased fitness of progeny [206]. Lima bean, *Phaseolus lunatus*, plants exposed to herbivore-induced plant volatiles experienced less herbivore damage along with possessing more leaves and living shoot tips [92,291]. Within the bioassay's timeframe of eight days, primed plants of either stage did not accumulate greater leaf biomass compared to caterpillar-infested control plants. In addition, because maize proceeds through morphologically distinct developmental stages, the duration of each stage was monitored. None of the treatments differed for stage duration. This is not surprising due to the severity of caterpillar infestation and resulting leaf tissue loss. At lower infestation levels and longer observation times, primed plants could exhibit greater tolerance.

JA may not indicate a primed defense response during caterpillar feeding

Typically, jasmonic acid induction indicates a response to caterpillar feeding and expression of direct and indirect defenses. It is thought that priming of herbivore resistance would involve or require the JA pathway [154]. Previous work, in 'Delprim' maize, found that simulated herbivory elicited more JA in primed compared to control plants [71]. However, caterpillar feeding did not yield a similar trend (Fig. 10). This does not exclude JA as a critical component of priming. The data suggests that JA levels may not predict a primed response in caterpillar-elicited maize. Confounding by non-equivalent damage in control and primed maize was avoided by starving the caterpillars for one hour prior to feeding. From a molecular perspective, a common criticism is that caterpillar feeding is a very strong stimulus that obscures a difference between elicited primed and non-primed plants. This may be true. However, I would argue that ten minutes of feeding only permits about one feeding site per larva. With an

infestation of three larva, the amount of damage produced in that short amount of time is less than or equal to the damage inflicted by a mechanical wounding tool.

Caterpillar feeding may not elicit enhanced volatile production in primed maize

Here, we report the characterization of the primed indirect defense response in maize due to caterpillar feeding. Plants experiencing herbivore damage synthesize and emit volatiles [297]. These volatiles can indirectly defend the plant by attracting predators and parasitoids [80,263]. Because sesquiterpene induction, in these maize genotypes, is seen by the second light cycle, the time course did not include multiple days. In general, volatile induction occurs twenty four hours after feeding begins [65,264,279,297]. Therefore, we chose this time course to evaluate whether caterpillar feeding elicits heightened volatile production in primed plants.

Primed maize plants did not produce elevated volatile amounts compared to control plants during caterpillar feeding (Fig. 11). Previous studies found that simulated herbivory elicited higher volatile production in primed maize plants [71,184]. However, it is difficult to draw any comparisons because of two key differences; elicitation occurred via larval feeding and the experiments were performed with a different maize genotype. Maize genotypes vary in volatile induction due to simulated herbivory and caterpillar feeding [72]. Therefore, it is not inconceivable that primed indirect defenses may also be genotype specific. Another possible explanation for a lack of elevated volatile induction in primed plants would be that caterpillar damage was less on primed plants. To mitigate this, plants were infested with several 3rd instar larvae. Preliminary bioassay data indicated that older instars were not as susceptible to primed plant tissue. In addition, observed damage did not differ between the treatments. From a molecular perspective, a common criticism is that caterpillar feeding is a very strong stimulus that

obscures a difference between elicited primed and non-primed plants. However, in an ecological context, volatile induction in primed maize during actual caterpillar feeding is most relevant.

In seedling stages, v1 and v3, volatile induction is not enhanced in primed plants during caterpillar feeding but these stages are limited in leaf area. Perhaps, primed volatile induction occurs in later stages with substantially greater biomass. To investigate this, volatiles were collected from primed and control v6 Tx601 plants infested with *S. exigua* larvae. Total volatiles released over twenty eight hours of feeding were not different between primed and control v6 plants (mean ± SE, ng g^{-1} FW, control fed 10485 ± 2436; primed fed 8020 ± 1428).

Equivalent volatile amounts do not necessarily exclude a primed indirect defense for two reasons. Parasitoid wasps may not respond to the dominant components of the volatile profile [84,265]. Unfortunately, our collection method limits us to C_5 or greater molecular weight compounds. Therefore, we were not able to assess other herbivore induced plant volatiles such as ethylene [113,159,298,299] and methanol [300]. Caterpillar feeding did not elicit elevated amounts of monoterpenes, sesquiterpenes, homoterpenes, green leafy volatiles, or indole in primed v1 and v3 plants compared to control v1 and v3 maize.

In a maize monoculture, plants grow in extremely high densities. In this way, it is an ideal situation for interplant priming to occur since volatiles may easily travel from one plant to another. If a caterpillar-infested neighbor is producing volatiles that act as signals to parasitoids and predators then it may be of little or no advantage to the undamaged neighbor to produce more volatiles once it is infested. In this case, an energetic advantage would be to the primed plant that invests in stronger, faster direct defenses and not indirect defenses.

Proteinase inhibitor transcript levels were not enhanced by caterpillars feeding on primed maize

Because of bioassay data showing reduced larval growth, it was expected that direct defenses may be elevated in primed maize plants. To evaluate direct defense expression, we measured proteinase inhibitor transcript levels. Previous research in the 'Delprim' hybrid measured enhanced transcript levels of several proteinase inhibitors in caterpillar-regurgitant treated primed versus control maize [184]. From this study, two 'priming non-responsive' and two 'priming responsive' genes were chosen. We measured transcript levels in v1 and v3 plants at six, twenty four, forty eight, and seventy two hours of caterpillar feeding. We chose this time course because bioassay data indicated that a delay in larval instar progression occurs within seventy two hours. Caterpillar-fed primed v1 and v3 plants compared to caterpillar-fed control plants did not contain higher transcript levels of any of the four PIs. First, transcript levels do not necessarily correlate to protein levels and protein levels do not always reflect activity. Second, a preliminary search of the maize genome produced eighty transcripts identified as a PI. Our comparative, v1 and v3, bioassay suggests that primed v1 plants reduce larval growth whereas v3 does not. These results suggest that the elicited, primed defense profile may vary by developmental stage. Of course, the possible effect of genotype-specific expression should be considered. However, in this case, perhaps, the faster, stronger response of primed maize does not rely on a few PIs and volatiles but actually is a broad induction of direct defenses with consistent indirect defense expression.

In summary, priming is a potentially useful strategy for plants to respond to and withstand environmental stresses while minimizing their energetic investment. Priming adds another subtle and complex layer to the mechanism of induced defenses. In this maize genotype, priming appears to limit growth of a generalist herbivore and consumption of leaf tissue. This

effect may be developmentally specific. Additionally, typical indicators of direct and indirect defenses did not reliably reflect the elicited, primed defense response. Extending our understanding of this phenomenon at mechanistic and ecological levels for a plant of intense agronomic importance is critical for the development of sustainable production strategies.

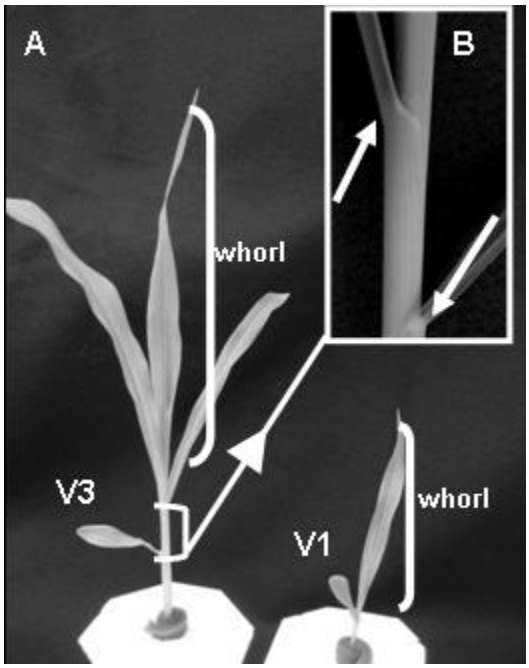

Figure 3-1: Image of V1 and V3 developmental stages. A) V1 and V3 stage plants with whorl portion indicated by brackets. B) Inset with arrows pointing to leaf collars, which are used to determine the developmental stage.

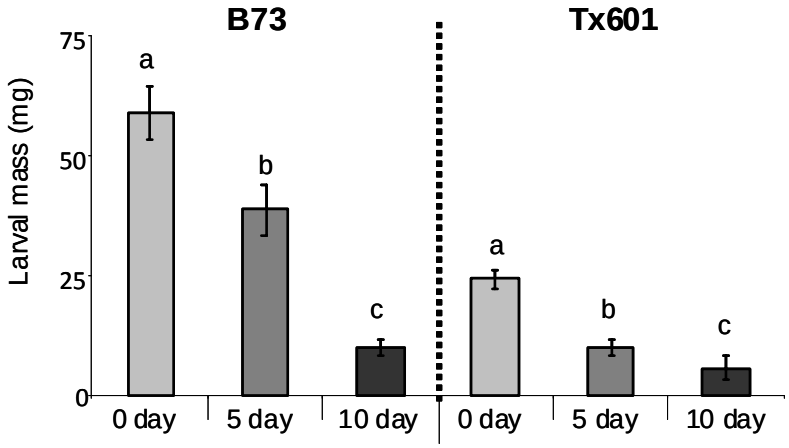

Figure 3-2: Comparison of larval performance from three levels of *Spodoptera exigua* egg stress feeding on excised tissue of two maize genotypes, B73 and Tx601 at the v1 stage. Eggs experienced ■ zero, ■ five, or ■ ten days of cold storage. Data represent mean ± SE of twenty five biological replicates per treatment after seven days of feeding. A two-way ANOVA indicated a significant interaction of genotype and egg stress ($F_{2,114}$=4.28, $p<0.016$). A one-way ANOVA was performed for each genotype. Egg stress was significant for B73 ($F_{2,57}$=50.97, $p<0.0001$) and Tx601 ($F_{2,57}$=40.67, $p<0.0001$). Comparisons for each genotype were conducted using the Tukey method with a family error rate of 0.05. Data were square-root transformed.

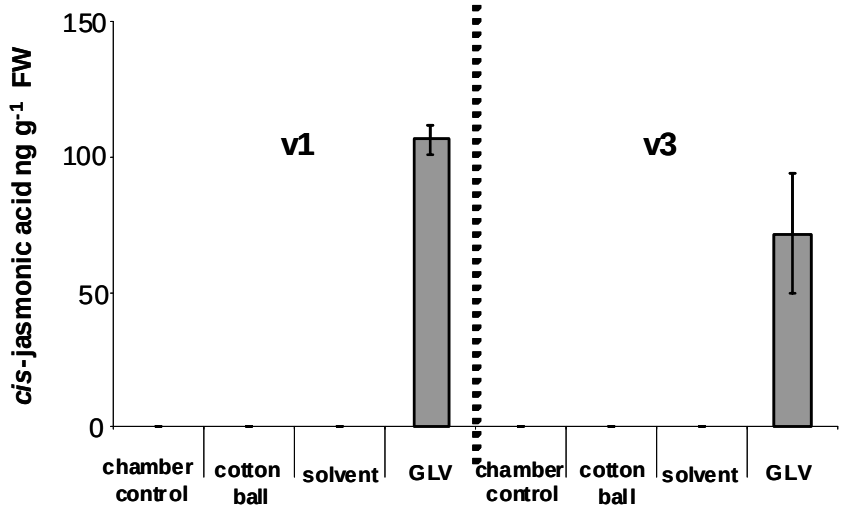

Figure 3-3: Comparison of transient JA burst in response to GLV exposure in v1 and v3 Tx601 maize plants. Maize, Tx601 inbred line, at v1 and v3 stages were exposed to (Z)-3-hexenyl acetate. Plants were treated by leaving the plant un-enclosed (chamber control), in a chamber with a clean cotton ball (cotton ball), in a chamber with the solvent, dichoromethane, deposited onto the cotton ball (solvent), or in a chamber exposed to (Z)-3-hexenyl acetate (GLV). The data represent amounts of cis-jasmonic acid measured in tissue samples from the developing leaves and four biological replicates per treatment. No cis-jasmonic acid was detected in any of the chamber, cotton ball, and solvent control samples. No significant differences were detected between v1 and v3 GLV-exposed plants (unpaired t-test, p=0.177).

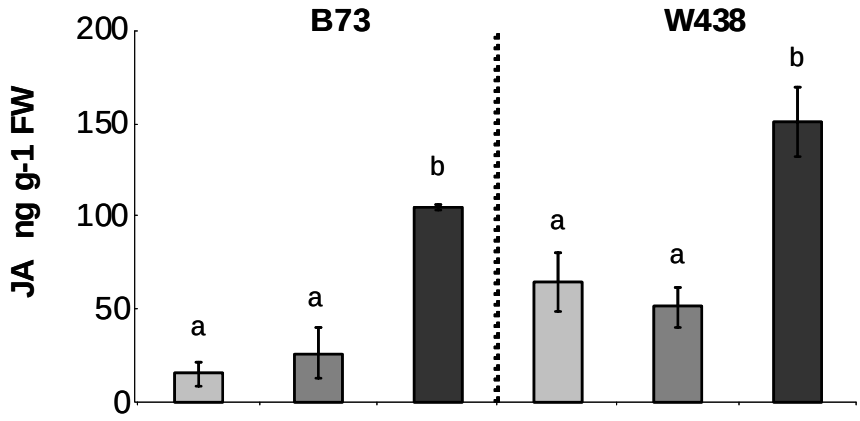

Figure 3-4: Comparison of transient JA burst in response to GLV exposure in two maize inbred lines. Maize inbred lines, B73 and W438, at the v1 stage were exposed to (Z)-3-hexenyl acetate or the solvent, dichloromethane. A control for the cotton ball and chamber was included. Data points correspond to ☐ control, ▩ solvent control, and ■ GLV. Each treatment represents three and four biological replicates, B73 and W438 respectively. A one-way ANOVA was used to compare treatments within a genotype. GLV-exposed plants were significantly different from controls in both genotypes (B73, $F_{2,6}=32.3$ $p<.0001$; W438, $F_{2,9}=12.67$ $p<.0002$). Post-hoc comparisons were performed for each genotype using the Tukey method at a family error rate of 0.05.

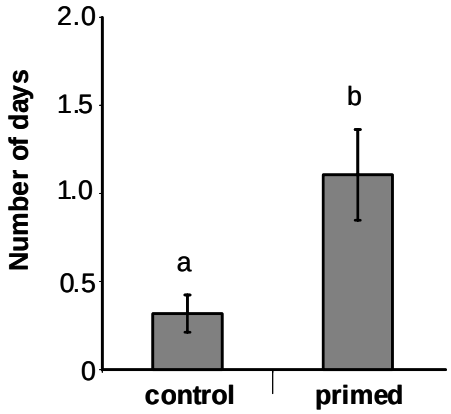

Figure 3-5: Comparison of instar duration for larvae feeding on excised control and primed v3 Tx601 maize leaf tissue. Data represent mean ± SE for number of days spent in the second instar. An unpaired, one-tailed t-test indicated significant differences between control and primed treatments ($t=-2.87$, d.f. 36, $p<0.01$). Each treatment had 16- 20 biological and the bioassay had three experimental replicates.

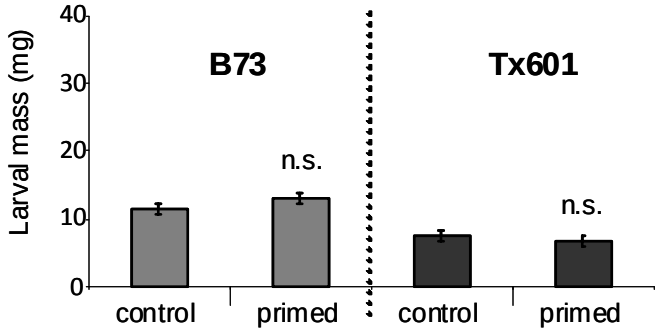

Figure 3-6: Comparison of larval performance on two primed maize genotypes. Larval weights were measured from beet armyworm larvae fed on excised control and primed leaf tissue from two maize genotypes, ▭ v1 B73 and ■ v3 Tx601. Beet armyworm, S. exigua, eggs experienced at least five days of cold storage. Data represent mean ± SE of thirty (B73) and twenty five (Tx601) biological replicates per treatment after six days of feeding. There were no significant differences between control and primed treatments (unpaired Student's t-test, B73, t=-1.29, d.f. 53, p=0.20; Tx601, t=0.74, d.f. 40, p=0.46). Data were square root transformed.

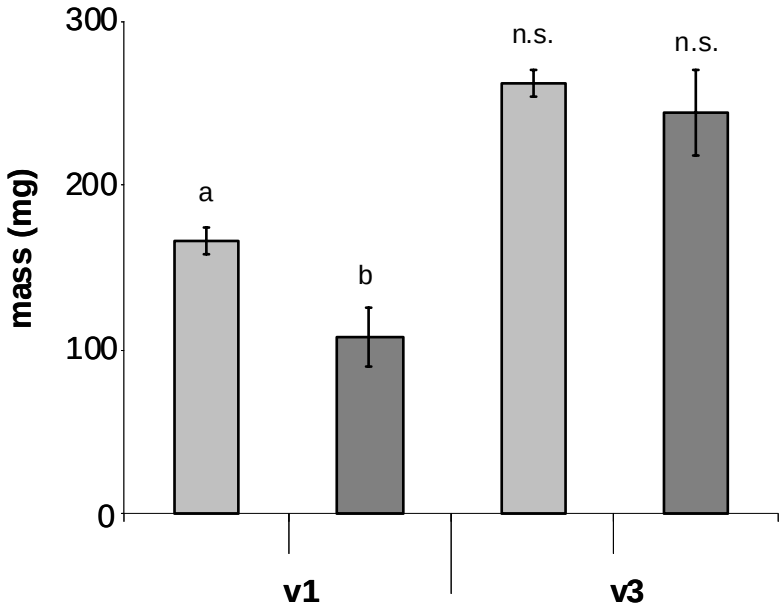

Figure 3-7: Larval performance on primed v1 and v3 maize. Total mass of larvae after seven days of feeding on intact ☐ control and ■ primed, v1 and v3, Tx601, maize. Data represent six biological replicates per treatment. Three experimental replicates were performed. Results reported for bioassay #1. Treatments were compared within a stage using an unpaired Student's t-test. Larvae masses were significantly different for v1 control and primed ($t=2.99$, d.f. 10, $p<0.014$) but not v3 ($t=0.65$, d.f. 10, $p<0.533$).

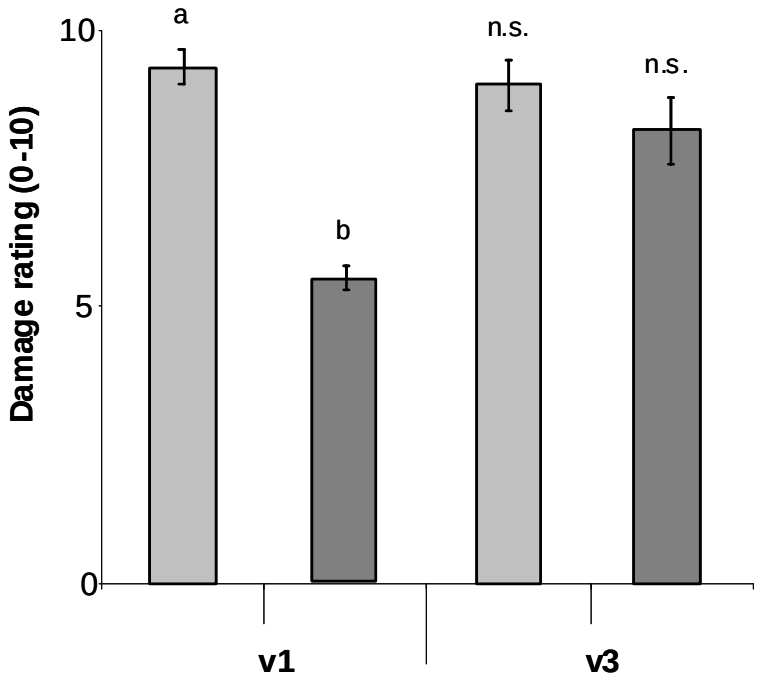

Figure 3-8: Damage rating of primed and control, v1 and v3 maize plants. Comparison of caterpillar-damaged ■ primed and □ control v1 and v3 Tx601 maize plants. Visual damage ratings were conducted using a modified method [282,283], see Methods for a description. A two-way ANOVA indicated a significant interaction of stage and treatment ($F_{1,19}=7.01$, $p<0.02$). Treatments were compared within a stage using an unpaired Student's t-test. V3 control and primed were not statistically different ($t=1.72$, d.f. 10, $p=0.12$). V1 control and primed were statistically different ($t=9.40$, d.f. 10, $p<0.0001$). Data represent six biological replicates per treatment. Three experimental replicates were performed. Results reported for bioassay #1.

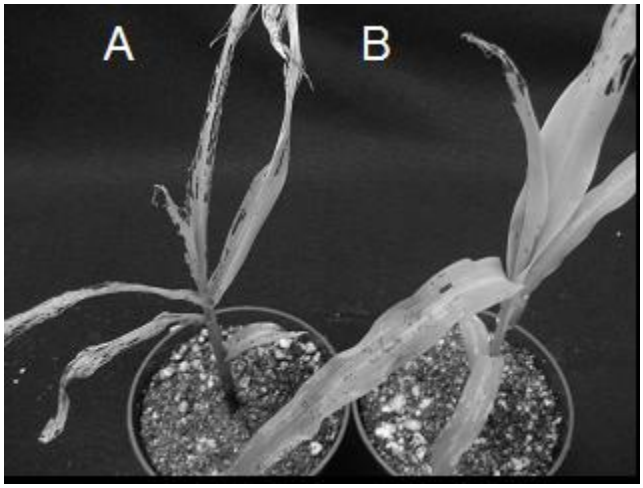

Figure 3-9: Caterpillar damaged v1 Tx601 maize plants after seven days of feeding. A) v1 control and B) v1 primed maize plant. These two plants experienced the least amount of damage in their respective groups.

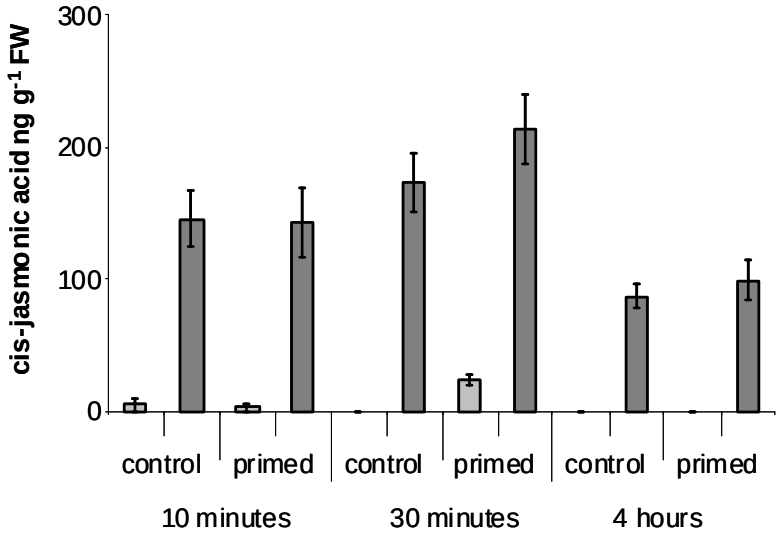

Figure 3-10: Comparison of beet armyworm damaged control and primed v3 Tx601 maize plants. Plants were exposed overnight to either (Z)-3-hexenyl acetate or dichloromethane, the solvent. Control and primed plants were either ☐ undamaged or ■ infested with larvae. Tissue was harvested at 10 minutes, 30 minutes, and 240 minutes. No significant differences were found for the comparison of control damaged and primed damaged maize at any of the time points (unpaired t-test: 10 minutes, 7 biological replicates, p=0.930; 30 minutes, 6 biological replicates, p=0.279; 240 minutes, 7 biological replicates, p=0.533). Note that each time point represents an independent experiment and therefore the JA levels cannot be compared between time points.

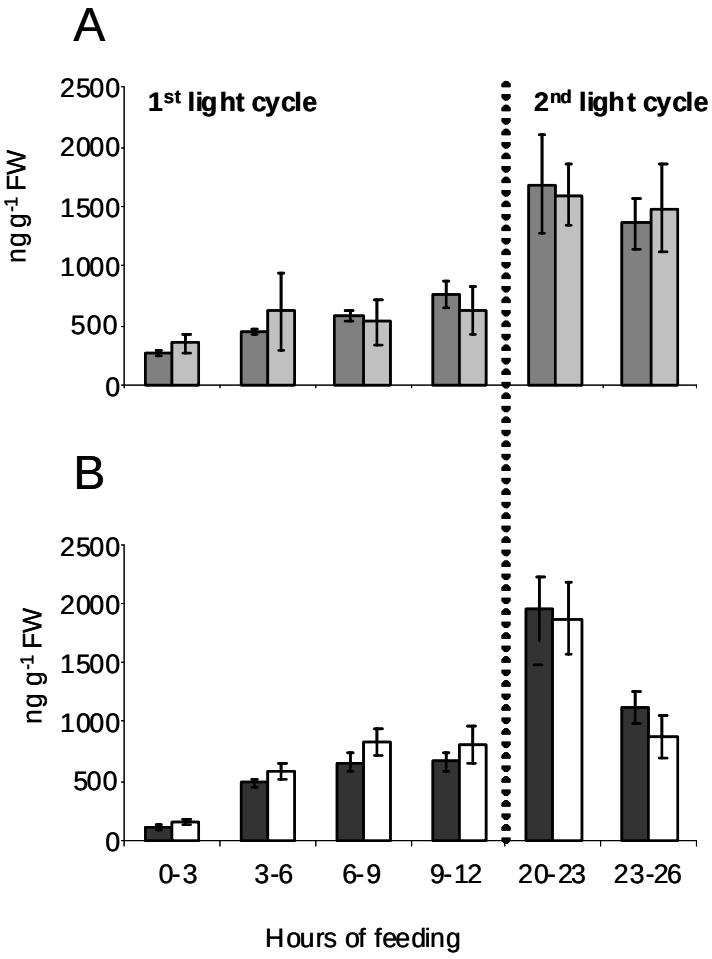

Figure 3-11: Volatile production in response to caterpillars feeding on primed and control, v1 and v3 maize. Time course of volatile production by A) v1 ☐ primed and ■ control maize B) v3 ☐ primed and ■ control maize. For both stages, plants were exposed overnight to either solvent (control) or (Z)-3-hexenyl acetate (primed). Plants were infested with beet armyworm larvae the following day. Volatiles were collected every three hours during the 1st and 2nd light cycles. Two plants per priming treatment were un-infested to verify induction due to feeding. Data points represent mean ± SE of four biological replicates for the total amount of volatiles collected over three hours and are normalized by fresh weight. All collection periods were during the light cycle. No significant differences were found for any of the time points (unpaired Student's t-test, α=0.05).

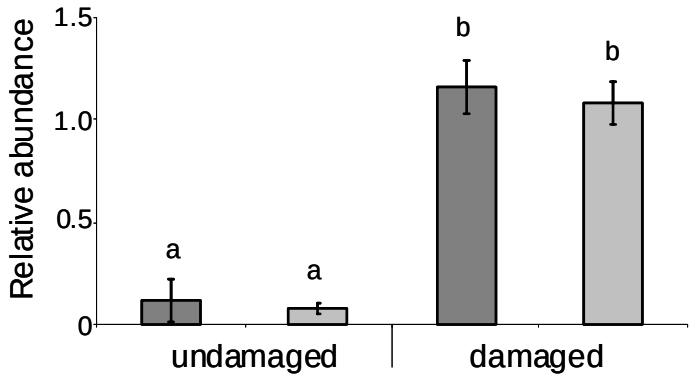

Figure 3-12: Cystatin-like proteinase inhibitor transcript accumulation after caterpillar feeding in v3 primed and control maize. Comparison of cystatin-like proteinase inhibitor transcript levels after thirty minutes of beet armyworm feeding on ■ control and □ primed v3 Tx601 maize. Plants were exposed overnight to either solvent (control) or (Z)-3-hexenyl acetate (primed). Plants were infested with starved beet armyworm larvae the following day. The larvae were permitted to feed for thirty minutes. Two plants per priming treatment remained undamaged to verify induction. Data points represent mean ± SE of six biological replicates for control damaged and primed damaged. Transcript levels were normalized to a reference gene. A two-way ANOVA indicated no significant differences of the priming treatment ($F_{1,12}=0.18$, $p=0.68$) or the interaction of priming and caterpillar feeding ($F_{1,12}=0.00$, $p=0.98$) . Caterpillar damaged plants were significantly different from undamaged controls ($F_{1,12}=59.68$, $p<0.0001$). Data were square-root transformed.

Table 3-1: Comparison of maize response to synthetic and natural GLV exposure. Amount (mean ± SE) of cis-Jasmonic acid in three genotypes of v1 maize after exposure to cut leaf tissue or (Z)-3-hexenyl acetate. Plants were treated in chambers with either a cotton ball (control), dichloromethane deposited onto a cotton ball (solvent), (Z)-3-hexenyl acetate (GLV), or 2 g of cut leaf tissue (leaf). After thirty minutes, plants were removed from the chambers and leaf tissue from the treated plant was harvested and analyzed for jasmonic acid.

GENOTYPE	CONTROL	SOLVENT	**LEAF**	**GLV**
B73	15 ± 7	26 ± 13	107 ± 15	115 ± 15
'Bonus'	0 ± 0	0 ± 0	65 ± 6	98 ± 11
W438	64 ± 16	51 ± 10	108 ± 14	135 ± 21

Table 3-2: Survival of larvae feeding on primed v1 and v3, Tx601, maize. Percent survival (mean ± SE) for beet armyworm, Spodoptera exigua, larvae feeding on intact primed and control plants of two developmental stages. Plants were primed by exposing each plant overnight to either solvent or (Z)-3-hexenyl acetate. Each treatment represents six replicate plants infested with ten neonates on day 1. The number of larvae on a per plant basis was recorded on day 7. Bioassays were replicated three times.

	V1 CONTROL	V1 PRIMED	V3 CONTROL	V3 PRIMED
BIOASSAY #1	57 ± 7	52 ± 10	67 ± 8	64 ± 9
BIOASSAY #2	57 ± 8	52 ± 5	68 ± 7	64 ± 7
BIOASSAY #3	55 ± 6	35 ± 3	44 ± 7	55 ± 6

Table 3-3: Average fresh weight of vegetative tissue in v1 and v3 primed and control Tx601 maize plants. Data represent mean ± SE of gram fresh weight for six biological replicates per treatment. Asterisk (*) denotes a significant difference between control and primed groups within a developmental stage, unpaired t-test (-3.77, d.f. 10, p=0.007). No other comparisons of primed and control groups by developmental stage were significantly different (Bioassay #1, v3 primed v. v3 control, -1.66, d.f. 10, p=0.129; Bioassay #2, v1 primed v. v1 control, -1.19, d.f. 10, p=0.262, v3 primed v. v3 control, -1.06, d.f. 10, p=0.312; Bioassay #3, v1 primed v. v1 control, -1.74, d.f. 10, p=0.112, v3 primed v. v3 control, 0.63, d.f. 9, p=0545).

	V1 CONTROL	V1 PRIMED	V3 CONTROL	V3 PRIMED
BIOASSAY #1	1.7 ± 0.2	2.5 ± 0.1 *	2.2 ± 0.3	2.9 ± 0.3
BIOASSAY #2	1.3 ± 0.3	1.8 ± 0.3	2.4 ± 0.6	3.2 ± 0.4
BIOASSAY #3	2.0 ± 0.2	2.5 ± 0.1	3.8 ± 0.4	3.4 ± 0.4

Table 3-4: Comparison of maize developmental stage duration between primed and control, Tx601, plants during an intact bioassay. Each plant was infested with ten neonate beet armyworm, Spodoptera exigua. Larvae were allowed to feed continuously for the next 7 days. Data represent mean ± SE of days in each stage and correspond to six biological replicates per treatment. No significant differences were found using an unpaired t-test when comparing control and primed treatments by developmental stage.

	V1 CONTROL	V1 PRIMED	V3 CONTROL	V3 PRIMED
V2 STAGE	4.0 ± 0.0	4.0 ± 0.0	NA	NA
V3 STAGE	4.7 ± 0.3	4.2 ± 0.4	1.8 ± 0.5	2.6 ± 0.7
V4 STAGE	0.3 ± 0.3	0.8 ± 0.4	7.2 ± 0.5	6.0 ± 0.6

Chapter 4

Conclusions and Future Directions

In chapter two, maize developmental stages, v1 and v3, express different defense profiles during simulated and actual herbivory. Simulated herbivory elicited less than 10 ng per g fresh weight of sesquiterpenes but did not elevate total volatile production in v1 maize. However, there is a small statistically significant induction of sesquiterpenes. To assess whether v1 plants responded to caterpillar regurgitant application, we measured jasmonic acid levels. Interestingly, v1 and v3 maize produced similar amounts of jasmonic acid thirty minutes after mechanical damage and addition of caterpillar regurgitant application. During caterpillar feeding, v1 and v3 maize produce the same amount of total volatiles when normalized for the amount of tissue. This suggests that the v1 maize defense response may require other factors present in the caterpillar's oral secretions or continuous mechanical damage to elicit volatile production.

On a per plant basis, v1 maize produced less than half the amount of volatiles compared to v3 in response to caterpillar feeding. We hypothesized that the biosynthetic pathway may be limited in either substrate availability or enzymatic activity. For this study, we assayed transcript amounts of farnesyl pyrophosphate synthetase that synthesized the substrate for terpene synthase 10, which is the last committed step of the sesquiterpene biosynthetic pathway. Transcript levels of farnesyl pyrophosphate synthetase were lower in caterpillar damaged v1 maize compared to caterpillar damaged v3 maize. In contrast, terpene synthase 10 was lower in caterpillar-damaged v3 plants compared to caterpillar-damaged v1 plants.

Proteinase inhibitor (PI) transcript levels were quantified to determine whether v1 maize engaged in a direct defense response. Herbivore-damaged v1 and v3 maize contained elevated amounts of all four PIs. A cystatin-like PI was higher in caterpillar damaged v1 maize compared

to damaged v3 maize. The results suggest that herbivore feeding induces a strong direct defense response along with a modest indirect defense response in v1 maize.

This is one of the few examples of an ontogenic characterization of induced defenses in a crop plant. For agricultural production, it is extremely important to understand how developmental stages influence a plant's ability to tolerate abiotic and biotic stresses present in its environment. This would allow for developmentally appropriate management strategies. For instance, it may be unnecessary to implement any control methods during a very young seedling stage because of its innate defenses. An additional study could consider characterizing defense responses of first generation European corn borer infestation at the whorl stage and later generations that infest other stages closer to the reproductive stages. Very little is known, at a molecular level, about defense induction by any economically important pests of maize. Bioassays could be performed to verify that v1 plants are more directly defended than v3 maize. Results from an intact bioassay (chapter 3) suggest that the solvent control v1 maize is more resistant to beet armyworm feeding than solvent control v3 maize. In addition, developing a reliable and reproducible assay for proteinase inhibitor activity would be an excellent step to confirm that transcript levels did in fact correlate with enhanced defense.

In chapter three, excised bioassays suggested that primed maize negatively affected neonates by delaying instar progression. Intact bioassay data indicate that primed v1 maize may effectively suppress larval growth compared to primed v3 maize. In the excised bioassay, priming did not reduce individual larval weights or relative growth rates after six days of feeding. During method development, it was discovered that a delay in instar progression in primed maize was only seen with neonates from eggs that had experienced cold stress. However, for intact bioassays, these "stressed" neonates would quickly die so, only eggs that had not experienced cold storage were used. In addition to reduced larval weights in the intact bioassay, larvae consumed less primed v1 maize tissue.

This study lays the foundation for defense characterization in primed maize. If primed maize did not negatively impact the herbivore then subsequent studies would be moot. The bioassays that I performed only assessed direct defense suppression of larval growth. A possible next step would be to perform parasitoid and predator assays. The two developmental stages, though important, are not typically infested with beet armyworm in this region and to the north. It would be important to determine the efficacy of priming for different herbivores at developmentally appropriate stages. Also, I did not extend the bioassay to the next generation to determine the fecundity and robustness of the larvae from parents that had fed on primed or control maize.

Also in chapter three, caterpillar feeding did not elicit higher volatile production, jasmonic acid levels, or proteinase inhibitor expression in primed maize compared to control plants. The primed state was verified by measuring jasmonic acid after thirty minutes of exposure to a green leaf volatile, z-3-hexenyl acetate. Caterpillar feeding at 10 minutes, 30 minutes, or 240 minutes did not elicit higher jasmonic acid production in primed v3 maize compared to control plants. The headspace of v1 and v3 primed and control maize plants was sampled for twenty two hours of caterpillar feeding. No differences were observed for the amount of total volatiles or leaf damage. Based on my observation of a delay in instar progression within the first seventy two hours of feeding, we measured proteinase inhibitor expression in v1 and v3 primed and control maize after six, twenty four, forty eight, and seventy two hours of feeding. Transcript levels were not higher in caterpillar damaged primed v1 or v3 maize compared to caterpillar damaged control plants at any of those time points. We evaluated an earlier time, at thirty minutes of feeding in primed and control v3 maize. No differences in transcript accumulation were observed.

These results suggest that the typical defense response indicators are not expressed at higher levels in caterpillar-damaged primed maize compared to caterpillar-damaged un-primed

control maize. However, bioassay data run counter to these results. There may be several reasons for this. Transcript levels do not necessarily correlate with protein abundance. The amount of protein does not always relate to activity. In this case, proteinase inhibitors (PIs) may not be responsible for reducing larval growth. Also, elevation of individual PIs may not be as effective as a broader activation of PIs along with other direct defenses such as induction of trichomes, deposition of leaf waxes, and toughening of tissue. Maize genotypes vary in their insect resistance. It would be interesting to select a few genotypes with well-characterized defense profiles and test whether priming of defense profiles varies by genotype. Perhaps, an extremely well-defended genotype doesn't utilize priming. Additionally, a study addressing whether herbivory in previous generations affected progeny's ability or propensity to prime would be of immense interest and value.

Priming is a complex and subtle process. Understanding the mechanisms of priming would reveal how plant defense induction occurs. My data suggest a re-consideration of primed maize defense profiles. Previous studies elicited primed maize with simulated herbivory. This allows for highly reproducible treatments and precise time courses but does not always resemble what happens when caterpillars are feeding. My finding that caterpillar feeding did not elicit greater volatile production in primed maize suggests that this maize genotype may not prime an indirect defense. Unfortunately, the lack of evidence for a primed direct defense response doesn't provide a complete story. However, this is perhaps the most valuable contribution of this work; to provoke further attempts to characterize and understand the primed defense profile in maize.

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