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Lessons from the Far End: Caterpillar FRASS-Induced Defenses in Maize, Rice, Cabbage, and Tomato

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Abstract
Plant defenses to insect herbivores have been studied in response to several insect behaviors on plants such as feeding, crawling, and oviposition. However, we have only scratched the surface about how insect feces induce plant defenses. In this study, we measured frass-induced plant defenses in maize, rice, cabbage, and tomato by chewing herbivores such as European corn borer (ECB), fall armyworm (FAW), cabbage looper (CL), and tomato fruit worm (TFW). We observed that caterpillar frass induced plant defenses are specific to each host-herbivore system, and they may induce herbivore or pathogen defense responses in the host plant depending on the composition of the frass deposited on the plant, the plant organ where it is deposited, and the species of insect. This study adds another layer of complexity in plant-insect interactions where analysis of frass-induced defenses has been neglected even in host-herbivore systems where naturally frass accumulates in enclosed feeding sites over extended periods of time.

Keywords: Frass, Caterpillar, Cabbage, Maize, Rice, Tomato

Introduction

Plants deploy a suite of induced and constitutive defenses that thwart insect attack (Chen 2008). Plant defense responses to insect herbivory have been attributed to several insect behaviors such as feeding, crawling, oviposition, and even defecation (Alborn et al. 1997; Felton and Tumlinson 2008; Hilfiker et al. 2014; Kim et al. 2012; Mithöfer et al. 2005; Peiffer et al. 2009; Ray et al. 2015). Insect feeding is associated with deposition of oral secretions and/or saliva on plant tissue, which leaves chemical cues of herbivory that induce defenses in host plants (Alborn et al. 1997, 2007; Musser et al. 2002; Schäfer et al. 2011; Schmelz et al. 2006). Induction of plant defenses in response to such chemical cues (or elicitors) is known to be specific to host-herbivore systems. For example, saliva from tomato fruit worm (TFW; *Helicoverpa zea*) induces herbivore defenses in tomato, but suppresses such defenses in tobacco (Musser et al. 2002; Tian et al. 2012). Induction of plant defenses differs even when the same elicitor is applied, such as glucose oxidase that suppresses direct defenses in tobacco, induces them in tomato, and has no effect in maize (Louis

et al. 2013b; Musser et al. 2002; Tian et al. 2012). Furthermore, insect secretions contain a blend of various molecules that differentially affect plant defense responses. For example, glucose oxidase from TFW saliva induces direct defenses in tomato, whereas salivary ATPases suppress them (Tian et al. 2012; Wu et al. 2012). Therefore, it is fair to say that the complexity of induced plant defenses in response to insect herbivory is highly specific to the composition of the herbivore secretion deposited on the plant, the plant species on which it is deposited, and the insect that deposits the secretion.

Our previous studies have shown that in the host-herbivore system of maize and fall armyworm (FAW; *Spodoptera frugiperda*), frass from the herbivore temporally induces pathogen defenses in maize whorl where it accumulates in contact with the feeding sites (Ray et al. 2015). However, there are several host-herbivore systems where frass does not accumulate in enclosed feeding structures over extended periods of time, and may only briefly come in contact with the wound site. We hypothesized that frass-induced defenses in plants are variable and specific to the host-herbivore system. To test our hypothesis, we measured both herbivore and pathogen-induced plant defenses in response to caterpillar frass. Since, caterpillar frass is more likely to accumulate in host-herbivore systems with enclosed feeding habits, we measured plant defenses in the following systems: European corn borer (ECB; *Ostrinia nubilalis*) frass in maize (*Zea mays*), cabbage looper (CL; *Trichoplusia ni*) frass in cabbage (*Brassica oleracea var. oleracea*), and TFW frass in tomato (*Solanum lycopersicon*) fruit. We also measured frass-induced plant defenses in host-herbivore systems where frass does not accumulate in close proximity to insect feeding sites, such as FAW frass on rice (*Oryza sativa*) leaf or TFW frass on tomato leaves. Although frass will not be present naturally in these host-herbivore systems for extended periods of time in nature, we measured plant defenses in response to frass in these systems over a four day period to compare these results with those from previous studies where frass accumulated in close proximity to feeding sites for extended periods of time (Ray et al. 2015).

Methods and Materials

Plant Material — Maize (*Zea mays* var. B73), tomato (*Solanum lycopersicum* var. Microtom), rice (*Oryza sativa* var. Nipponbare), and white cabbage (*Brassica oleracea var. oleracea* Platinum dynasty) were grown in glasshouse conditions with 16:8 h L:D cycle. Maize seeds were germinated in Promix-HP potting mix (Premier Tech Home and Garden, Ontario, Canada) and 1-wk.-old seedlings were transplanted in field soil until they reached the V8 stage (Ritchie et al. 1998). Cabbage and tomato plants were grown to their desired stages in Promix-HP potting mix containing mycorrhizae. Rice seeds were germinated first on filter paper with 16:8 h L:D cycle and then transplanted in Metro-mix 360 (Sungro, MA, USA). All plants were fertilized with Osmocote Plus (Scotts, OH, USA) once after potting, and rice plants additionally were fertilized with 5% ammonium sulfate solution weekly, and once with slow release iron chelator 1 % Sprint 330 (Hummert International, MO, USA) after potting. Maize seeds (var. B73) were obtained from USDA-ARS in Mississippi State University, USA; rice seeds were obtained from USDA-ARS Dale Bumpers National Rice Research Center in Arkansas; white cabbage seeds (var. Platinum dynasty) were purchased from Seminis (MO, USA).

Insects, Collection and Extraction of Frass — Fall armyworm, cabbage looper, and European corn borer larvae were reared on rice, white cabbage, and maize leaves, respectively, in plastic cups. Frass from the larvae was collected each day, plastic cups were cleaned, and fresh plant tissue was given to the larvae each day. Tomato fruitworm larvae were fed both on tomato leaves and green fruits in plastic cups. Frass was collected each day, and fresh tissue supplied to the larvae for feeding. Frass obtained from the different larvae species was homogenized in 1×–phosphate-buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate) for 30 min at a ratio of 1 g (wet weight) of frass in 5 ml of PBS. The homogenized frass slurry then was filtered through Miracloth (EMDMillipore, USA) to separate the insoluble debris. The soluble frass extract was filter sterilized using a 0.2-µm filters (EMD Millipore, USA). The sterilized frass extracts were concentrated using a 3-kDa molecular weight cut-off column (Pall Life Science, USA). Protein concentrations in the frass extract were measured using a Bradford protein quantification assay with bovine serum albumin as standard (Bradford 1976). Twenty micrograms of total frass protein were applied to each wound site on leaves of the plants or injected into tomato fruit.

Treatment of Plant Material with Frass Extract — Whorl leaves of V8-stage maize plants were treated with frass or buffer on wound sites as described previously (Ray et al., 2015). Rice plants were grown for 30 d after germination when plants typically had three tillers. The leaves of all tillers were wounded with a hole-punch, and frass proteins or buffer was applied. Cabbage plants were grown for 30 d to the four-leaf stage, then the leaves were wounded with the maize wounding tool (Ray et al. 2015) and treated with either frass proteins or buffer. Tomato plants were grown for 4 wk (4-leaf stage), leaves were wounded with hole-punch, and then treated with frass proteins or buffer. Fruit treatments were done on unripe tomato fruits on 6-wk.-old plants by injecting frass proteins or buffer into the fruit using a 10-µl pipette tip.

RNA Extraction and cDNA Preparation — Leaf and fruit tissues were homogenized in liquid nitrogen using GenoGrinder 2000 (OPS Diagnostics, USA) and RNA was extracted with TRIzol reagent (Life Technologies, USA) using the manufacturer's protocol (100 mg tissue in 1 ml TRIzol). Genomic DNA was removed from the extracted RNA by treating it with 2.5 M lithium chloride overnight at 4°C. The precipitated RNA was washed with 75 % ethanol twice and re-suspended in nuclease-free water. The genomic DNA-free RNA was quantified by using a Nanodrop (Thermo-Fisher Scientific, USA). One microgram of total RNA was then used to prepare cDNA using the Olido-dT with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). RNA extraction and cDNA synthesis were performed for each biological replicate separately. Four biological replicates for each treatment were used for maize plants, 5 for each treatments in cabbage and tomato plants, and 7 replicates for each treatment in rice per time point.

Quantitative Real-Time PCR — Quantitative real-time PCR (qRT-PCR) was performed with Fast Start Universal SYBR Green Master Mix (Roche Applied Science, USA). Gene-specific primers were designed with Primer Express 3.0 (Life Technologies, USA; details in Table 1). Relative quantification (RQ) of the target gene expression was calculated by the delta-delta Ct method (Livak and Schmittgen 2001) using *actin* as an endogenous gene for maize and rice, *ubiquitin* for tomato, and *glyceraldehyde-3-phosphate dehyrdogenase* for cabbage. Gene expression levels in frass-treated, PBS buffer-treated and undamaged control plants were measured 4, 24, 48, and 96 h after application for the respective target genes tested.

Trypsin Protease Inhibitor Assay — Trypsin protease inhibitor (TPI) assay was performed on tomato leaves and fruits treated with 20 µg of frass for 4, 24, 48, and 96 h. One hundred milligrams of plant tissue were ground in GenoGrinder (OPS Diagnostics, USA) as described above and homogenized in 1.25 ml of extraction buffer (0.046 M Tris HCl pH = 8.1, 0.012Mcalcium chloride) containing 5% insoluble polyvinyl-pyrrolidone (Chung and Felton 2011). Samples were centrifuged at 11,000 g for 10 min, and 10 µl of the supernatant were mixed with 80 µl of extraction buffer and 10 µl of 1 mM trypsin (Sigma Aldrich, USA). The mixture was incubated at room temperature for 10 min, and 100 µl of the substrate (2 mM p-toluene-sulfonyl-L-arginine methyl ester, Sigma Aldrich) were added, and the optical density (OD) was measured for 5 min at 247 nm. TPI activity per milligram of protein was calculated by the formula, PI = [1-(A/B)]/P where A represents the trypsin activity of the sample, B represents the maximum trypsin activity in a sample where only extraction buffer was added (no inhibitor present), and P is milligram of protein added to measure TPI activity. The protein concentration of each sample was measured separately by Bradford assay using bovine serum albumin as a standard (Bradford 1976).

Insect Bioassays — The leaflet of the two oldest leaves of 4-wk.- old tomato plants were treated with TFW frass fed on tomato leaves. Each leaflet was wounded with a cork borer and treated with 20 µg of frass protein. Fifteen plants were treated with frass and another fifteen were treated with equivalent volume of buffer in a similar manner. Leaf tissue were harvested from frass-treated, buffer-treated, and undamaged tomato leaves after 24 h of treatment and fed to first instar TFW caterpillars. Caterpillar weights were measured at the start of the bioassay and after 4 d. Relative growth rate (RGR) of the caterpillars at the end of 4 d was measured (Mohan et al. 2008). To perform bioassays with the tomato fruits, frass collected from TFW feeding on tomato fruits was injected to green fruits. Twenty microgram of frass protein or an equivalent volume of buffer were injected into tomato fruits with a micropipette as described earlier. Fruits from 15 plants were each treated with frass, buffer, or left undamaged. Fruits were harvested and fed to first instar TFW larvae and their RGR was measured.

Data Analyses — RQ values for gene expression and TPI activity were analyzed with a two-factor ANOVA using time and treatment (frass, buffer, or undamaged controls) as independent variables with SAS 9.2 (SAS Institute Inc., USA) software at $P < 0.05$ level of confidence. However, there were interactions between time and treatment for all genes/TPI activity tested, hence a multiple comparison Tukey test was performed for each gene at $P < 0.05$ level of confidence for each set of host and insect-frass systems tested. A one-way ANOVA was performed after normalization of the RGR data for insect bioassays at $P < 0.05$ and means separation was performed by Tukey's test ($P < 0.05$).

Results

European Corn Borer Frass Sustains Herbivore-Induced Defenses while Suppressing Pathogen-Induced Defenses in Maize — European Corn Borer herbivory is known to induce defenses in crops such as maize and tomato (Houseman et al. 1992; Louis et al. 2013a). Oral secretions and saliva of ECB have been implicated in harboring elicitors (and/or effectors) that trigger direct and indirect herbivore defenses in plants (Louis et al. 2013a, b). However, defenses triggered by the presence of elicitors (and/or effectors) from ECB frass have not been studied. ECB larvae feed in enclosed host tissues such as the maize stem or the maize whorls where frass may accumulate in close proximity of fed tissue over extended periods of time. We measured the transcript abundance of herbivore-induced *lipoxygenase3* (*Zm-lox3*) and *maize protease inhibitor* (*Zm-mpi*)

Table 1. List of primers used for quantitative-real time PCR (qRT-PCR)

Gene	Plant Species	Forward	Reverse	NCBI Accession Number
Actin	Maize	GGAGCTCGAGAATGCCAAGAGCAG	GACCTCAGGGCATCTGAACCTCTC	U60511.1
Lipoxygenase 3 (Zm-lox3)	Maize	GCTACGTACGAGCTGGTACATGAA	GCCGCTCTCTTCCCGTTT	AF149803
Maize Protease Inhibitor (Zm-mpi)	Maize	GCGGATTATCGCCCTAACC	CGTCTGGGCGACGATGTC	X78988
Pathogenesis-related gene 5 (Zm-pr5)(Erb et al. 2009)	Maize	TGCATGCATGGGCTAGTGAT	CGCACACAAATCCAGCTACG	U82201
Actin	Rice	ATCCTGACGGAGCGTGGTTA	TAGTCCAGGGCGATGTAGGAA	NM_001057621.1
Lipoxygenase (Os-RCI-1)	Rice	TATCCCATCCCCATCCACTTAT	GTGTGAATGATTTGCAGCTGAAC	AJ270938.1
Bowman-Birk Protease Inhibitor (Os-RPI)	Rice	CGTTCGATCATTCAGAGTTGGTATA	AAGCATGCAAGATGCACAAAA	AB098712.1
Non-expressor for pathogenesis related protein 1 (Os-NPR1)	Rice	CTTTTGGATCTCGCACTTGCA	CCTCGCAGCAATGTGAAGAA	DQ450948.1
Glyceraldehyde-3-phosphate dehydrogenase (gapdh)	Cabbage	GGTGGTGCGAAGAAAGTT	AGTGGACGGTGGTCATAA	EF123055.1
Lipoxygenase(Bo-Lox)	Cabbage	CTTGCTAAGACTCACGCTATT T	GCGTTGACGAGACTTTG	EF123056.1
Trypsin protease inhibitor (Bo-Tpi)	Cabbage	CTGCGCTCAGTCAACTTAT	GCAATCGTTACCGTCTCTAC	EU126815.1
Pathogenesis related protein 1 (Bo-Pr1)	Cabbage	CAGCCCTTGTAGGAGCTCTTGT	GGTTGTGAGCGTTTACATAGTCTTG	EF423806.1
Ubiquitin	Tomato	GCCAAGATCCAGGACAAGGA	GCTGCTTTCCGGCGAAA	X58253
Lipoxygenase D (Sl-Lox D)	Tomato	GTTCATGGCCGTGGTTGACACATT	TGGTAATACACCAGCACCACACCT	U37840
Protease inhibitor 2 (Sl-Pin2)	Tomato	GGATTTAGCGGACTTCCTTCT G	ATGCCAAGGCTTGTACTAGAGAATG	K03291
Pathoegenesis related protein 1-p4 [Sl-PR1-(P4)]	Tomato	TGTCTCATGGTATTAGCCATATTTCACT	CGTTGTGAACCGCAAGATAGTC	AJ011520

in response to ECB frass proteins at 4, 24, 48, and 96 h in maize leaves. We also measured pathogen-induced *pathogenesis-related defense protein5* transcript (*Zm-pr5*) abundance in leaves treated with ECB frass proteins at the same time points (van der Linde et al. 2012; van Loon et al. 2006). *Zm-lox3* encodes *lipoxygenase3* in the jasmonic acid (JA) biosynthesis pathway, and is a hallmark for herbivore-induced early defense in maize. Frass-treated leaves showed higher abundance of *Zm-lox3* transcripts compared to controls at 4 h, however, frass treatment suppressed transcript abundance at 48 h (Fig. 1a). Another herbivore-induced defense gene *Zm-mpi*, showed higher transcript accumulation in leaves treated with frass at 4, 24, and 96 h compared to control (Fig. 1b). Notably, the marker for pathogen defense *Zm-pr5* showed higher transcript levels only at 24 h in frass-treated maize leaves; however, frass treatment suppressed *Zm-pr5* abundance at 48 and 96 h (Fig. 1c). This suggests that frass-induced plant defenses in maize may be insect-specific since these results are in contrast to the results obtained with FAW frass that increased the expression of *Zmpr5* in maize between 8 and 48 h (Ray et al. 2015).

Cabbage Looper Frass Triggers an Oscillating Pattern of Herbivore Defenses in Cabbage over Time — We measured caterpillar frass-induced defenses in cabbage by CL frass proteins. This represents another naturally occurring plant-herbivore host-plant system where frass is likely to accumulate in an enclosed structure. Transcript abundance of herbivore defense-related genes such as *lipoxygenase* (*Bo-Lox*) that is involved in the JA biosynthesis pathway in the Brassicaceae and induced by caterpillar herbivory was measured (Zheng et al. 2007).We also determined the transcript levels of *trypsin protease inhibitor* (*Bo-Tpi*) that acts downstream of the JA pathway and is known to retard CL growth in cabbage (Broadway and Colvin 1992). Transcript abundance of both *Bo-Lox* and *Bo-Tpi* in response to CL frass proteins and buffer at 4, 24, 48, and 96 h were measured. The JA biosynthesis precursor *Bo-Lox*, showed higher transcript abundance in frass-treated plants only at the early time point of 4 h compared to buffer-treated plants (Fig. 2a). After 4 h, *Bo-Lox* transcripts steadily declined in response to frass treatment at 24 and 48 h and were not different between frass- and buffer-treated plants at 96 h. However, *Bo-Lox* transcript levels in both buffer- and frass-treated plants increased dramatically at 96 h, but were not different from each other. *Bo-Tpi* transcript accumulation showed an oscillating pattern of induction and suppression in response to frass- treatment. Transcript levels of *Bo-Tpi* in frass-treated plants were higher than buffer-treated plants at 4 h, whereas they were suppressed at 24 h. Then, the frass-treated plants showed higher induction of *Bo- Tpi* transcripts at 48 h, followed by suppression at 96 h (Fig. 2b).

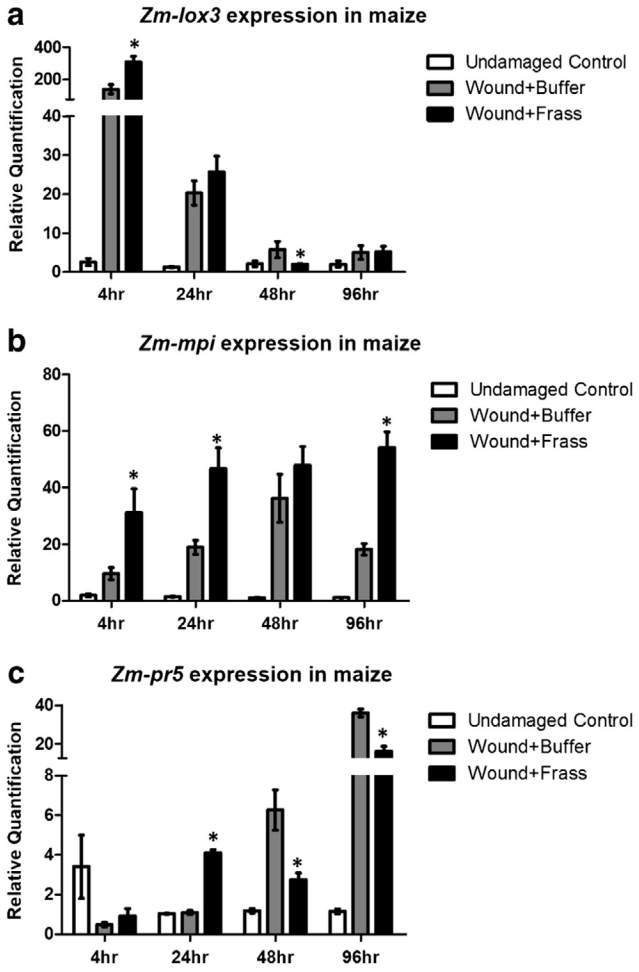

Fig. 1. Maize defense gene expression in response to European corn borer frass. Maize leaves (var. B73) were wounded and treated with either frass proteins or PBS buffer or left undamaged for 4, 24, 48, and 96 h. Relative expression (RQ) of *lipoxygenase3* (*Zm-lox3*) **(a)**, *maize protease inhibitor* (*Zm-mpi*) **(b)** and *pathogenesis-related protein5* (*Zmpr5*) **(c)** were measured by quantitative real-time PCR (qRT-PCR) by normalizing transcript abundance to that of the reference gene *actin*. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey's test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants ($P < 0.05$) at the respective time points. Error bars indicate standard error of the mean

Pathogen attack causes the induction of the *pathogenesis-related protein1* (*Bo-Pr1*) gene in cabbage (Park et al. 2005). Therefore, we measured *Bo-Pr1* transcript abundance in response to CL frass proteins in cabbage (Fig. 2c). The *Bo-Pr1* transcript levels were slightly induced at 4 h and suppressed at 24 h compared to buffer-treated plants (Fig. 2c). However, the expression of *Bo-Pr1* transcripts increased dramatically at 48 and 96 h, and was significantly higher than the buffer-treated controls (Fig. 2c). The induction of *Bo-Pr1* at later time points suggest that there could be a shift to an enhanced pathogen defenses by CL frass in cabbage over time.

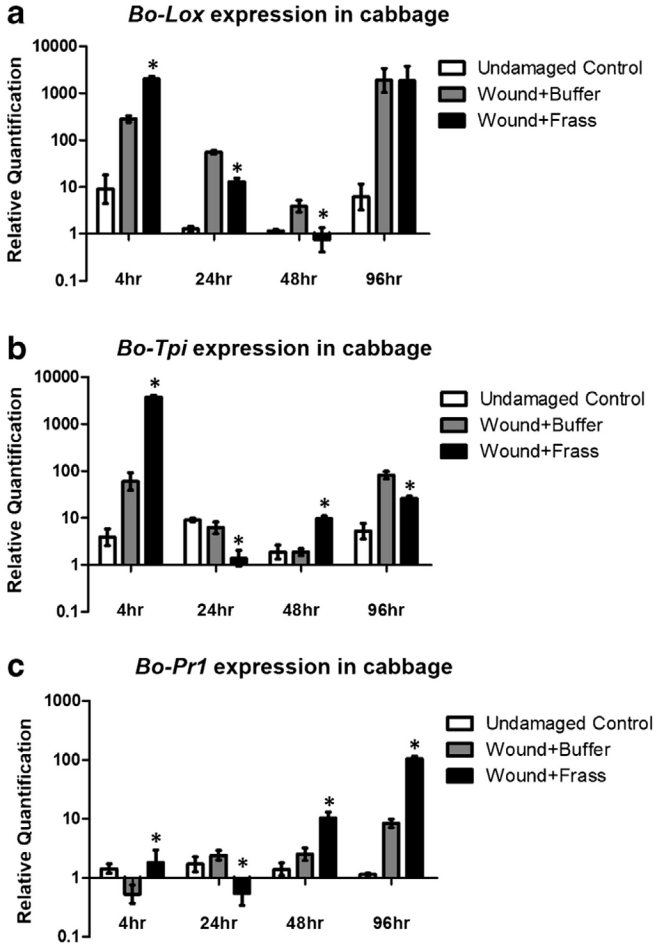

Fig. 2. Defense gene expression in cabbage in response to cabbage looper frass. Cabbage (var. Platinum dynasty) plants were wounded and treated with either frass proteins or PBS buffer or left undamaged for 4, 24, 48, and 96 h. Relative expression (RQ) of *lipoxygenase* (*Bo-Lox*) **(a)**, *trypsin protease inhibitor* (*Bo-Tpi*) **(b)** and *pathogenesis-related protein1* (*Bo-Pr1*) **(c)** were measured by quantitative real-time PCR (qRT-PCR) by normalizing transcript abundance of target genes to that of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase*. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey's test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants ($P < 0.05$) at the respective time points. Error bars indicate standard error of the mean

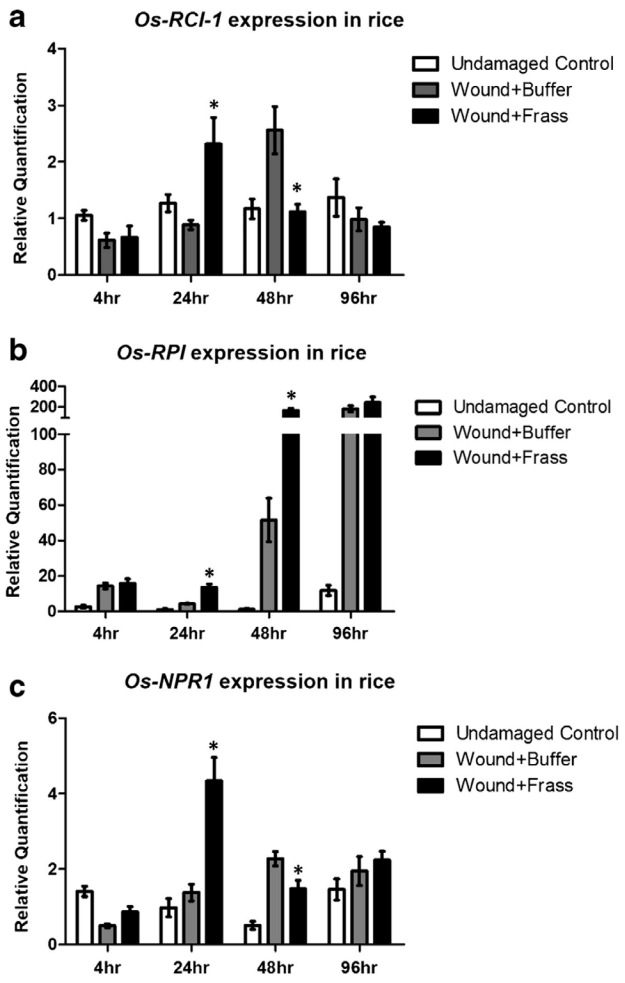

Fig. 3. Defense gene expression in rice in response to fall armyworm frass. Rice (cv. Nipponbare) plants were wounded and treated with either frass proteins or PBS buffer or left undamaged for 4, 24, 48, and 96 h. Relative expression (RQ) of rice *lipoxygenase* (*Os-RCI-1*) **(a)**, *rice protease inhibitor* (*Os-RPI*) **(b)** and *non-expresser of pathogenesis-related protein1* (*Os-NPR1*) **(c)** were measured by quantitative real-time PCR (qRT-PCR) by normalizing transcript abundance to that of *actin*. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey's test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants ($P < 0.05$) at the respective time points. Error bars indicate standard error of the mean

Fall Armyworm Frass Steadily Induces Herbivore Defenses in Rice while Suppressing Pathogen Defenses — Fall Armyworm is a generalist herbivore that feeds on several important crop species including rice (Ali and Agrawal 2012; Pashley 1986). We have shown that frass proteins from FAW caterpillars fed on maize trigger a pathogen defense response when they deposit their frass in the enclosed feeding sites of the whorls (Ray et al., 2015). Here, we measured defenses triggered by frass proteins from FAW larvae that fed on rice. The rice-FAW interaction is a host-herbivore system where frass does not accumulate in close proximity to feeding sites, but falls off from the leaves during herbivory. Jasmonic acid biosynthesis-related lipoxygenase gene (*Os-RCI-1*) expression is induced by *Spodoptera litura* feeding in rice and JA treatment (Schaffrath et al. 2000; Xu et al. 2003).We observed that *Os-RCI-1* had higher transcript abundance in frass protein-treated plants only at 24 h after application (Fig. 3a). However, at 48 h after application, frass-treated plants showed a suppression of *Os-RCI-1* transcript abundance compared to the controls (Fig. 3a). A Bowman-Birk rice protease inhibitor (*Os-RPI*) downstream in the JA signaling pathway has been shown to be induced by beet armyworm (*Spodoptera exigua*) herbivory in rice (Venu et al. 2010). Transcript abundance of

herbivore-induced *Os-RPI* was weakly induced in frass-treated plants compared to controls at 24 h, but it increased dramatically in frass-treated plants at 48 h (Fig. 3b). Rice shows a strong SA-JA crosstalk and is known to regulate SA-JA antagonism through *non-expresser of pathogenesis-related protein1* (*Os-NPR1*) that is an early marker for the salicylic acid (SA) pathway and plays a critical role in pathogen defense in rice (Chern et al. 2005; Thaler et al. 2012; Yuan et al. 2007). In addition, overexpression of *Os-NPR1* in rice increases its susceptibility to herbivores (Yuan et al. 2007). When we measured *Os-NPR1* transcript abundance in response to FAW frass in rice, the frass-treated plants showed a higher abundance of *Os-NPR1* transcripts than the controls at 24 h. However, at 48 h when the herbivore-induced *Os-RPI* transcript peaked, *Os-NPR1* transcript abundance was suppressed (Fig. 3c). These data strongly suggest that FAW frass proteins trigger sustained herbivore defenses while suppressing pathogen-induced defenses in host-herbivore system where frass does not accumulate in close proximity to feeding sites.

Leaf-Fed Tomato Fruitworm Frass Induces Sustained Herbivore Defenses in Tomato Leaves — As another example of a host-herbivore system where frass does not accumulate in host organs, we measured defenses in tomato leaves in response to frass proteins from TFW that also were fed on tomato leaves. *Lipoxygenase D* (*Sl-Lox D*), involved in JA biosynthesis, and *Protease inhibitor 2* (*Sl-Pin2*) downstream of the JA pathway are markers of herbivore-induced defense genes in tomato (Peiffer et al. 2009; Tian et al. 2012). Leaf-fed TFW-frass proteins caused weak suppression and a subsequent induction of *Sl-Lox D* transcripts at 4 and 48 h, respectively, compared to buffer-treated tomato leaves (Fig. 4a). However, *Sl-Pin2* transcript abundance increased appreciably in frass-treated leaves at all time points (Fig. 4b). We also measured transcript abundance of a SA-induced pathogen defense gene *pathogenesis related protein 1-p4* [*Sl-PR1-(P4)*] in response to frass protein on leaves (Chung et al. 2013). Transcript abundance of *Sl-PR1-(P4)* was same in both frass- and buffer-treated tomato leaves at all time points tested (Fig. 4c). These results provide strong evidence that when leaf-fed TFW-frass proteins are applied on a tomato leaf, a site where frass does not accumulate near the feeding sites, the frass proteins induce herbivore defenses without triggering pathogen defenses as seen with ECB, CL, or FAW frass (Figs 1c, 2c, 3c).

Fruit-Fed Tomato Fruitworm Frass Shows an Initial Suppression Followed by an Induction of Herbivore Defenses in the Fruit — To better understand if frass-induced defenses are altered when TFW feeds in the enclosed tomato fruit, we collected frass proteins from TFW fed on fruit, injected it into the fruit, and then measured the same defense gene transcripts as for tomato leaves. The

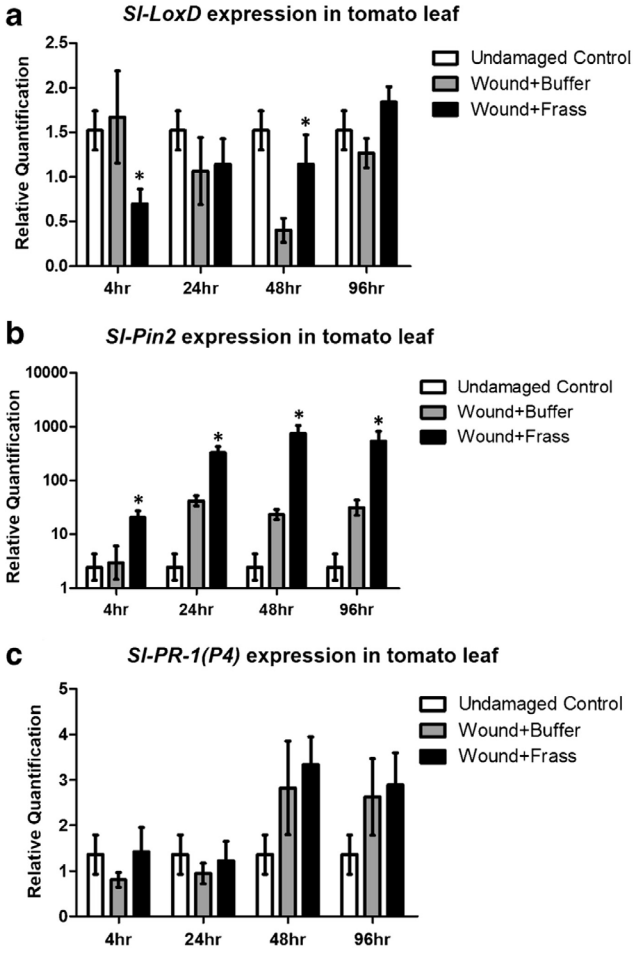

Fig. 4. Defense gene expression in tomato leaves in response to tomato fruitworm frass fed on tomato leaves. Tomato (var. Microtom) leaves were wounded and treated with either frass proteins or PBS buffer or left undamaged for 4, 24, 48, and 96 h. Relative expression (RQ) of *lipoxygenaseD* (*Sl-LoxD*) **(a)**, *protease inhibitor2* (*Sl-Pin2*) **(b)** and *pathogenesis-related protein1 (p4)* (*Sl-PR1-(P4)*) **(c)** were measured by quantitative real time PCR (qRT-PCR) by normalizing transcript abundance to that of the house-keeping gene *ubiquitin*. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey's test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants ($P < 0.05$) at the respective time points. Error bars indicate standard error of the mean

herbivore-induced JA biosynthetic marker *Sl-Lox D* showed higher transcript levels in frass-treated plants compared to controls only at the later time points of 48 and 96 h (Fig. 5a). However, expression of the downstream JA-induced herbivore defense gene *Sl- Pin2* was suppressed in frass-injected tomato fruits compared to controls at 4 and 24 h. On the other hand, *Sl-Pin2* RNA levels were higher in TFW frass-injected fruits at 48 and 96 h (Fig. 5b). Compared to buffer-treated fruits, frass treatment suppressed the pathogen-induced SA marker gene *Sl- PR1-(P4)* at 24 and 48 h after frass injection. In contrast, frass treatment induced higher *Sl-PR1-(P4)* transcript levels at 96 h as compared

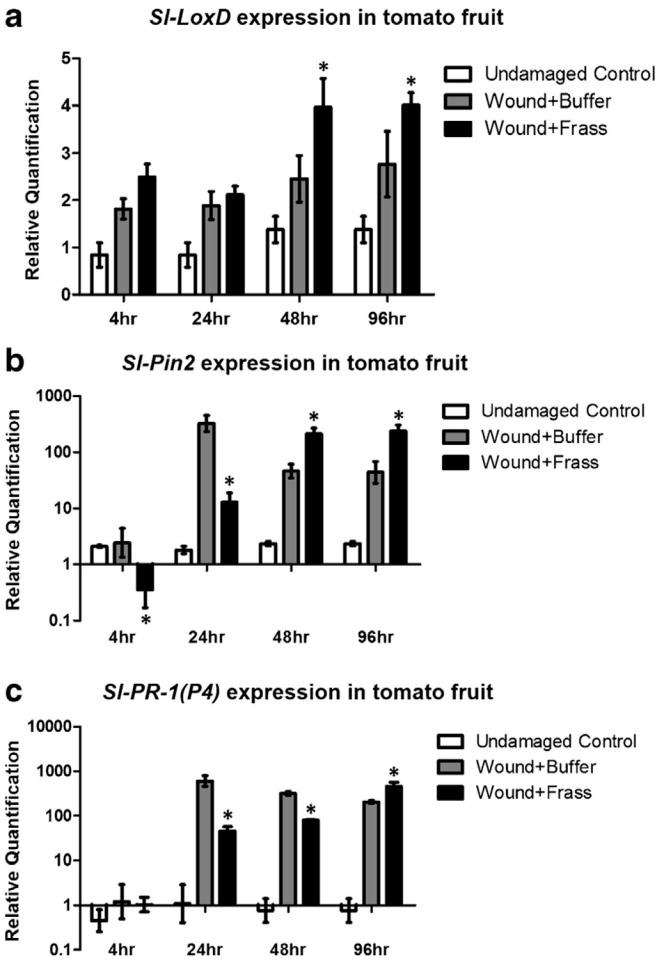

Fig. 5. Defense gene expression in tomato fruits in response to tomato fruitworm frass fed on tomato fruits. Tomato (var. Microtoms) fruits were injected with either frass proteins or PBS buffer or left undamaged for 4, 24, 48, and 96 h. Relative expression (RQ) of *lipoxygenaseD* (*Sl-LoxD*) **(a)**, *protease inhibitor2* (*Sl-Pin2*) **(b)** and *pathogenesis-related protein1 (p4)* (*Sl-PR1-(P4)*) **(c)** were measured by quantitative real-time PCR (qRT-PCR) by normalizing transcript abundance to that of the housekeeping gene *ubiquitin*. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey's test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants ($P < 0.05$) at the respective time points. Error bars indicate standard error of the mean.

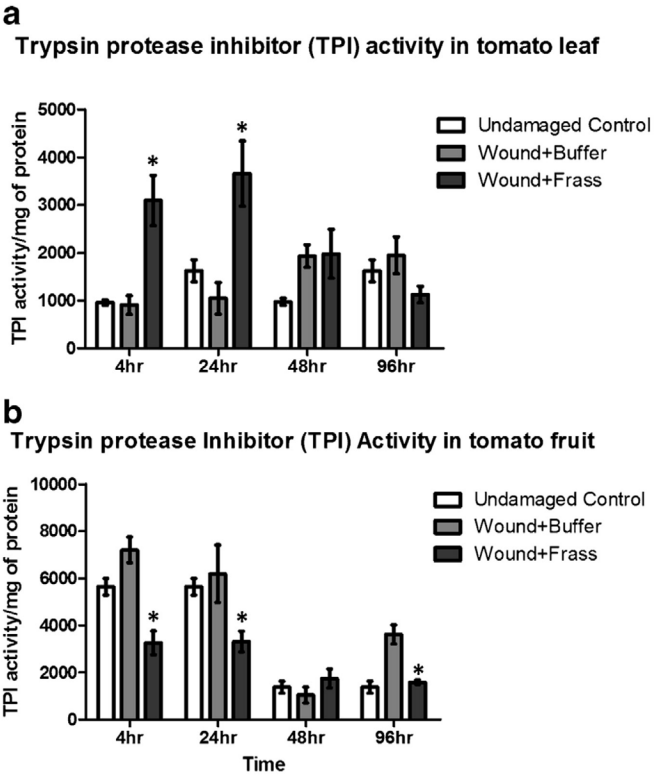

Fig. 6. Trypsin protease inhibitor (TPI) activity in tomato leaves and fruits in response to tomato fruitworm frass (TFW) fed on tomato leaves and fruits, respectively. Tomato (var. Microtoms) fruits were injected with either frass proteins or buffer and leaves were treated with frass proteins or buffer or left undamaged for 4, 24, 48, and 96 h. TPI activity was measured by spectrophotometer in both leaves **(a)** and fruits **(b)** and expressed as TPI activity per milligram of protein. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey's test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants ($P < 0.05$) at the respective time points. Error bars indicate standard error of the mean.

to buffer-treated fruits (Fig. 5c). Taken together, the results from TFW frass treatment on tomato leaves and fruits suggests that the frass-induced defense response depends not only on the diet of the defecating herbivore, but also on the organ/tissues where they deposit their frass.

Tomato Fruitworm Frass Induces Trypsin Protease Inhibitor Activity in Tomato Leaves, but Suppresses it in Fruits — In an attempt to understand if changes in defense gene transcript abundance affect the biochemistry of the plant organs that are treated with frass proteins, we measured trypsin protease inhibitor (SI-TPI) activity in tomato fruits and leaves in response to TFW frass. Protease inhibitors are induced in response to insect herbivory in a number of plants, and they prevent the digestion of proteins in the insect gut, thereby increasing the demand for essential amino acids and retarding insect growth (Chung and Felton 2011; Felton 2005). These two host-herbivore interactions represent examples of systems where frass accumulates and remains in the enclosed fruit or where it is briefly in contact with feeding sites on the leaves. Previously, it was shown that SI-TPI activity followed the similar pattern as of *Sl-PIN2* transcript abundance after insect herbivory on tomato (Chung and Felton 2011). Our results indicate that SI-TPI activity was higher in leaves treated with tomato leaf-fed frass at 4 and 24 h compared to controls (Fig. 6a). However, after 24 h, SI-TPI activity was the same in both frass and buffer-treated leaves. Similarly, in fruits injected with frass from TFW fed on fruits, the TPI activity was lower compared to fruits injected with buffer at 4, 24, and 96 h (Fig. 6b). SI-TPI activity was induced in leaves, but suppressed in fruits at 4 and 24 h, and these results followed

the same overall trend in *Sl-PIN2* transcript induction at these time points (Fig. 4b, 5b).We conclude that both *Sl-PIN2* gene and protein expression was altered in response to frass application.

Performance of Tomato Fruitworm Larvae Is Enhanced in Frass-Treated Tomato Fruits but Reduced in Frass-Treated Tomato Leaves Compared to Buffer-Treated Plants — Since we observed frass-treated tomato leaves showed an induction of herbivore defenses at 24 h as indicated by *PIN2* gene expression and protease inhibitor assay (Fig. 4b, 6a), we fed leaf-fed TFW frass-treated and buffer-treated tomato leaves for 24 h to first instar tomato fruitworm larvae. TFW larvae grew slower when they consumed frass-treated leaves compared to buffer-treated or undamaged leaves (Fig. 7a). Similarly, we also fed TFW larvae tomato fruits treated with fruit-fed TFW frass for 24 h since frass suppressed herbivore defenses in the fruits at this time (Fig. 5b, 6b). When TFW larvae were fed tomato fruits treated with frass, the caterpillars grew faster compared to those treated with buffer (Fig. 7b). These bioassay results validate the biochemical and the gene expression data, which show that tomato leaf-fed TFW frass, when applied to tomato leaves, induces herbivore defenses that in turn reduce the performance of the herbivore on the leaves. However, tomato fruit-fed TFW frass when injected to tomato fruits, suppresses herbivore defenses that enhance the performance of the herbivore on the tomato fruits.

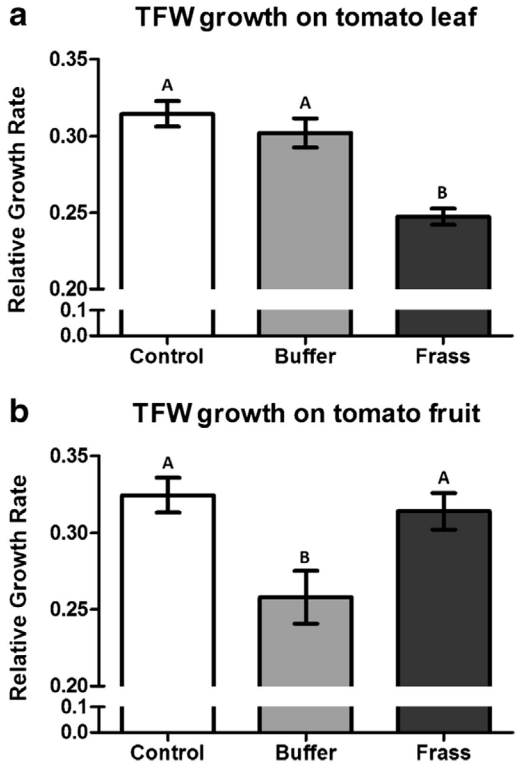

Fig. 7. Effect of tomato fruitworm (TFW) frass-treated tomato leaves and fruits on the growth rate of naïve TFW caterpillars. Leaf-fed TFW frass or buffer was applied to tomato leaves and fruit-fed TFW frass or buffer was injected to tomato fruits for 24 h. Tissues were collected from frass-, buffer-treated plants and undamaged control plants and fed to naïve first instar caterpillars. Relative growth rate (RGR) of the caterpillars feeding on leaves **(a)** or fruits **(b)** were measured after 4 d. RGR values with different letters are significantly different from each other according to Tukey's mean separation ($P < 0.05$). Error bars indicate standard error from the mean.

Discussion

Herbivore-induced defenses can be specific to insect cues depending on the plant species and the insect depositing cues on the plant (Acevedo et al. 2015; Karban and Baldwin 1997). The repertoire of plant defense compounds that are induced in response to herbivory on tobacco from the tobacco hornworm (*Manduca sexta*) and beet armyworm (*Spodoptera exigua*) are different (Voelckel and Baldwin 2004). Alternatively, herbivore cues such as saliva from the same insect TFW suppresses herbivore defenses in tobacco, but induces them in tomato (Musser et al. 2002; Tian et al. 2012). Although, most of these studies are focused on caterpillar oral secretions or saliva, little is known about frass-induced plant defenses in various host-herbivore systems. This is of particular importance in understanding the complexity of host-herbivore interactions, since the composition of frass can change depending on the plant tissue consumed by the herbivore. Furthermore, the host's response to frass could also vary depending on the organ where it is deposited and the duration of time it remains on the plant.

Our previous study demonstrated that in a host-herbivore system such as FAW and maize where frass accumulates in the whorl over time, herbivore defenses are suppressed and pathogen defenses are induced (Ray et al. 2015). In this study, we measured the transcript abundance of both pathogen- and herbivore-induced genes in three additional systems where frass accumulates in the host's enclosed organs, viz., ECB frass in the maize whorl, CL frass on cabbage leaves, and TFW frass inside the tomato fruit. We measured herbivore induced *lipoxygenase3* (*Zm-lox3*) and *maize protease inhibitor* (*Zm-mpi*) transcript abundance, both of which are induced by caterpillar herbivory in maize (Chuang et al. 2014; Louis et al., 2013b), in response to ECB frass on maize leaves. Similar to FAW frass-induced defenses in maize, ECB frass also induced higher levels of *Zm-lox3* and *Zm-mpi* transcripts compared to buffer-treated plants 4 h after application (Fig. 1a, b). However, *Zm-mpi* transcript abundance was higher in plants treated with ECB frass at 24 and 96 h compared to controls (Fig. 1b). On the other hand, the pathogen-induced *Zm-pr5* transcript levels were higher only at 24 h and then suppressed compared to controls at 48 and 96 h (Fig. 1b, c). This result is in contrast to our previous study where FAW frass caused a temporal shift in defenses from herbivore to pathogen defenses in maize and *Zm-pr5* transcript abundance steadily increased after 24 h of frass application (Ray et al., 2015).

Cabbage looper also feeds in relatively enclosed spaces on cabbage leaves, and their frass remains in contact with wounds on the host leaves for long periods. Our study revealed that pathogen-induced *Bo-Pr1* transcript levels steadily increased at 48 and 96 h after frass application (Fig. 2c). Notably, the herbivore-induced JA precursor *Bo-Lox* showed reduced transcript levels at both 24 and 48 h in response to frass treatment (Fig. 2a). Another herbivore-induced gene transcript, *Bo-Tpi*, showed an oscillating pattern of induction at 4 h (and 48 h) followed by suppression at 24 (and 96 h) that is reminiscent of the Z-scheme model of effector-triggered immunity. The Z-scheme is a widely accepted model in plant-pathogen interactions, and it is implicated to be of importance in plant-herbivore interactions as well (Felton and Tumlinson 2008; Jones and Dangl 2006). In general, CL frass treatment suppressed herbivore defenses while activating pathogen defenses. This was similar to the pattern found in the TFW-tomato fruit interaction where frass also accumulates in an enclosed host organ. Fruit-fed TFW frass injected into fruits induced a temporal shift towards pathogen defenses by inducing *Sl-PR1-(P4)* transcript accumulation in fruits at 96 h (Fig. 5c). This is in contrast to the expression of *Sl-Pin2*, a marker for herbivore-induced defense response. *Sl-Pin2* transcript abundance was suppressed at 4 and 24 h in frass-injected fruits, and this was mirrored by suppressed TPI activity at these time points (Fig. 5b, 6b). At 48 and 96 h, *Sl-Pin2* transcript levels were higher compared to the buffer-injected tomato fruits, however this was not reflected in the Sl-TPI activity in fruits at these time points (Fig. 6b). Although CL frass on cabbage leaves and TFW frass in tomato fruits activated the pathogen-defense pathway, we cannot conclude that all host-herbivore systems where frass accumulates in enclosed host feeding sites can induce a pathogen-defense pathway since this was not observed in maize when ECB frass was applied on maize leaves (Fig. 1 a-c).

As examples of host-herbivore systems where frass comes in contact with the host plant for a limited amount of time, we measured frass-induced defenses of FAW on rice and TFW frass on tomato leaves. In rice, the transcript abundance of herbivore-induced *lipoxygenase* (*Os-RCI-1*) increased in response to FAW frass at 24 h and then was suppressed at 48 h (Fig. 3a). Transcript abundance of *rice protease inhibitor (Os-RPI),* a gene that is further downstream of *Os-RCI-1* in the herbivore-defense pathway, was slightly higher in frass-treated plants at 24 h, but dramatically increased at 48 h compared to buffer-treated plants (Fig. 3b). Interestingly, the pathogen-induced *Os-NPR1* gene was suppressed in response to frass at 48 h when *Os-RPI* was at its peak (Fig. 3c). This is contrary to frass-induced defenses of the same herbivore (FAW) in maize where pathogen defenses were induced over time (Ray et al. 2015). In tomato leaves, leaf-fed TFW frass consistently induced higher abundance of the herbivore-induced *Sl-Pin2* transcripts from 4 to 96 h (Fig. 4b). The *Sl- Pin2* transcript

Table 2. Summary of frass-induced defenses in different host-herbivore systems

Host plant tissue on which frass is deposited	Insect species	Host tissue consumed by insect to generate frass	Enclosed feeding habit?	Herbivore defenses triggered over time?	Pathogen defenses triggered over time?
Maize leaves (*Zea mays* var. B73)	European corn borer (*Ostrinia nubialis*)	Maize leaves	Yes	Yes	No
Cabbage leaves (*Brassica oleracea* var. oleracea)	Cabbage Looper (*Trichoplusia ni*)	Cabbage leaves	Yes	No	Yes
Rice leaves (*Oryza sativa* var. Nipponbare)	Fall armyworm (*Spodoptera frugiperda*)	Rice leaves	No	Yes	No
Tomato leaves (*Solanum lycopersicum* var. Microtom)	Tomato fruitworm (*Helicoverpa zea*)	Tomato leaves	No	Yes	No
Tomato fruit (*Solanum lycopersicum* var. Microtom)	Tomato fruitworm (*Helicoverpa zea*)	Tomato fruits	Yes	No	Yes
Maize leaves (*Zea mays* var. B73)	Fall armyworm (*Spodoptera frugiperda*)	Maize leaves	Yes (Ray et al. 2015)	No (Ray et al. 2015)	Yes (Ray et al. 2015)

abundance also correlated with higher protease inhibitor activity at 4 and 24 h in frass-treated samples (Fig. 6a). Furthermore, the pathogen defense marker in tomato *Sl-PR1-(P4),* which was induced in response to TFW frass in fruits, was not induced on leaves at any of the time points (Fig. 4c, 5c). These results are particularly interesting since leaf-fed TFW frass induces the herbivore defenses in leaves, while the frass from the same herbivore when fed on fruits induces antagonistic pathogen defenses in tomato fruits. This is further demonstrated by the slower performance of TFW larvae on tomato leaves when treated with leaf-fed TFW frass compared to buffer-treated controls (Fig. 7a). Contrastingly, TFW caterpillars grew much faster on fruits that were injected with fruit-fed TFW frass compared to buffer-treated controls (Fig. 7b). Taken together these data strongly suggest that frass-induced defenses are specific to host-herbivore systems and differ even when the same herbivore species feed on different host organs or tissues.

Herbivore frass is composed of a complex blend of biomolecules arising from the insect, host plant, and microbes present in the gut or frass (Chen et al., 2005, Chen et al., 2007; Ray et al., 2015). We have only begun to understand how endophytic symbionts in the insect gut can alter plant defenses (Chung et al., 2013). Similarly, little is known about herbivore frass-induced defenses in plants (Ray et al., 2015; Schwartzberg and Tumlinson 2014). In this study, we present an overview of how frass-induced defenses can alter host defenses depending on the insect depositing the frass, the host plant and the organ where the frass is deposited (Table 2). Frass composition is likely to change depending on the host organ/tissue where the herbivore feeds. Such change in frass composition could alter the herbivore-associated cues deposited on the host. Another level of added complexity for frass-induced defenses is the recognition of herbivore cues by the host. Induction of plant defenses can be tissue-specific (Erb et al., 2012; Karban and Baldwin 1997), which could possibly explain the contrasting effects of TFW frass-induced defenses in tomato fruits and leaves (Figs. 5, 6). Finally, frass-induced defenses appear to be temporally regulated and can change from herbivore-defense induction to pathogen defense-induction as the time of frass exposure increases. This was demonstrated in the case for CL frass-induced defenses on cabbage. In other cases, there was either a sustained herbivore defense (TFW frass applied to tomato leaves) or sustained pathogen defense (TFW frass injected into tomato fruit). Taken together, we conclude that frass-induced defenses on host plants are extremely complex and specific to the host-herbivore system. Considerable work needs to be done to understand the mechanism of frass-induced defense elicitation in plants.

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