Ethylene Signaling Modulates Herbivore-Induced Defense Responses in the Model Legume *Medicago truncatula*

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One or more effectors in the labial saliva (LS) of generalist Noctuid caterpillars activate plant signaling pathways to modulate jasmonate (JA)-dependent defense responses; however, the exact mechanisms involved have yet to be elucidated. A potential candidate in this phytohormone interplay is the ethylene (ET) signaling pathway. We compared the biochemical and molecular responses of the model legume *Medicago truncatula* and the ET-insensitive *skl* mutant to herbivory by fourth instar *Spodoptera exigua* (Hübner) caterpillars with intact or impaired LS secretions. Cellular oxidative stress increases rapidly after herbivory, as evidenced by changes in oxidized-to-reduced ascorbate (ASC) and glutathione (GSH) ratios. The caterpillar-specific increase in GSH ratios and the LS-specific increase in ASC ratios are alleviated in the *skl* mutant, indicating that ET signaling is required. Ten hours postherbivory, markers of the JA and JA/ET pathways are differentially expressed; *MtVSP* is induced and *MtHEL* is repressed in a caterpillar LS- and ET-independent manner. In contrast, expression of the classic marker of the systemic acquired resistance pathway, *MtPRI*, is caterpillar LS-dependent and requires ET signaling. Caterpillar LS further suppresses the induction of JA-related trypsin inhibitor activity in an ET-dependent manner. Findings suggest that ET is involved in the caterpillar LS-dependent, salicylic acid/NPR1-mediated attenuation of JA-dependent induced responses.

Although the jasmonate (JA)-mediated signaling pathway primarily governs plant induced resistance (IR) in response to chewing insect herbivores, ethylene (ET) signaling cascades modulate this response in either a positive or negative manner (Zhu 2014). ET, induced by the oral secretions of the tobacco hornworm *Manduca sexta*, suppresses nicotine biosynthesis in *Nicotiana attenuata* (Voelckel et al. 2001). Therefore, some insect herbivore species have evolved one or more mechanisms to counteract plant IR by exploiting this hormonal crosstalk (Felton and Korth 2000). One or more effectors in insect saliva may activate antagonistic or synergistic phytohormone pathways to modulate the plant's JA-dependent IR (Diezel et al. 2009; Eichenseer et al. 2010; Musser et al. 2006; Paudel et al. 2013; Walling 2008; Weech et al. 2008); this is believed to be a strategy evolved by some generalist Noctuid caterpillar species to circumvent plant defenses.

As caterpillars feed, plant tissues are macerated and mandibular and labial saliva (LS) are released onto the wounded tissue. Through both the actual wounding and enzymes present in the LS, caterpillar herbivory leads to the generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), around the wound site (Arimura et al. 2011; Maffei et al. 2006, 2012; Orozco-Cárdenas et al. 2001). At low, controlled levels, H_2O_2 is an important second messenger that leads to the activation of JA-mediated signaling pathways (Arimura et al. 2011; Forman et al. 2010). However, at higher levels, H_2O_2 is detrimental to cellular function, as it can lead to lipid peroxidation and protein oxidation as well as reacting with nucleic acids (Møller et al. 2007). Thus, the induction and activation of enzymes such as peroxidases and catalases are critical to detoxify ROS (Maffei et al. 2006; Quan et al. 2008). Also, to balance the cellular redox levels, the Halliwell-Asada (ascorbate/glutathione [ASC/GSH]) cycle is activated (Noctor et al. 2012; Quan et al. 2008). In this cycle, cellular H_2O_2 levels are lowered through a series of reduction-oxidation reactions that involve ASC and GSH; ASC peroxidase catalyzes the reduction of H_2O_2 to water, with the subsequent oxidation of ASC to dehydroascorbate (DHA), via the intermediate monodehydroascorbate (Foyer and Noctor 2011). DHA reductase reduces DHA to ASC, with the subsequent oxidation of GSH to generate GSSG. This last metabolite is reduced back to GSH by glutathione reductase generating $NADP^+$ from $NADPH$. Besides maintaining cellular redox homeostasis, GSH is also involved in defense signaling cascades directly or as a result of protein posttranslational modifications, such as glutathionylation or S-nitrosylation (Han et al. 2013; Spoel and Loake 2011).

In response to biotic stresses, changes in oxidative stress are observed either by increases in the cellular redox metabolic pool or by shifting the ratio of oxidized-to-reduced redox metabolites (Noctor et al. 2012). Total GSH levels often increase in plants infected by pathogens or treated by salicylic acid (SA), leading to the activation of the systemic acquired resistance (SAR) pathway (Mateo et al. 2006; Mou et al. 2003; Petersen et al. 2000). Therefore, plants compromised in their ability to synthesize or accumulate GSH are more susceptible to pathogens and insect herbivores (Ball et al. 2004; Maughan et al. 2010; Parisy et al. 2007; Schlaeppi et al. 2008). For example, the *Arabidopsis pad2-1* mutant that accumulates one-fifth of wild-type GSH levels due to a mutation in the gene encoding γ -glutamylcysteine synthetase (γ -GCS), the enzyme that catalyzes the first step of GSH biosynthesis, is more vulnerable to herbivory by caterpillars of the Egyptian cotton leafworm, *Spodoptera littoralis* (Parisy et al. 2007; Schlaeppi et al. 2008). Apart from changes in the total GSH pool, a shift in the ratio of GSSG/GSH is also important in stress responses (Noctor et al. 2012). An increased ratio of GSSG/GSH and, in particular, an increase in GSSG levels is correlated with the

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activation of the JA defense pathway (Mhamdi et al. 2010). It was, therefore, unexpected that, in *Arabidopsis*, foliar herbivory by caterpillars of the beet armyworm *S. exigua* had no effect on total cellular GSH levels or the GSSG/GSH ratio within the first 45 min (Paudel et al. 2013). Also, at 35 min, GSSG levels are reduced. In contrast, if caterpillar LS secretion is impaired, total GSH pool levels are lower compared with control plants or plants infested by caterpillars with normal labial salivary secretions. This suggests that caterpillar LS plays a role in modulating the plant cellular oxidative state in response to insect feeding. In fact, generalist noctuid caterpillars, such as *S. exigua*, have abundant oxidoreductases in their LS, such as glucose oxidase (GOX) that oxidizes glucose to produce gluconate and H₂O₂ (Bede et al. 2006; Eichenseer et al. 2010; Musser et al. 2002).

Changes in the cellular oxidative state are closely linked to the activation of the plant's defense responses. For example, GSSG accumulation activates JA biosynthesis, leading to the degradation of JA-Zim proteins and the subsequent release of MYC2/3/4 transcription factors that regulate JA-dependent gene expression (Cheng et al. 2011; Chini et al. 2007; Dombrecht et al. 2007; Fernández-Calvo et al. 2011; Lorenzo et al. 2004; Mhamdi et al. 2010; Pauwels et al. 2010; Staswick and Tiryaki 2004; Thines et al. 2007; Xu et al. 2002; Yan et al. 2009). Thus, JA-mediated IR becomes active, inducing expression of JA-related defense genes such as polyphenol oxidase (PPO) and trypsin inhibitor (TI) that interfere with the nutrient availability needed for insect growth (Constabel and Barbehenn 2008; Felton 2005; Zhu-Salzman et al. 2008).

Input from other phytohormone signaling pathways that modify the JA signaling cascade are also regulated through changes in the cellular oxidative state. For example, increased GSH levels leads to the activation of the nonexpressor of pathogenesis-related protein1 (NPR1) in the SA-dependent SAR pathway that can lead to the attenuation of JA-dependent IR (Koornneef et al. 2008; Ndamukong et al. 2007; Spoel et al. 2003). In addition, changes in cellular ROS and GSH affect ET signaling pathways that can either enhance or attenuate JA-regulated defense responses, in part, by modulating the SA-mediated attenuation of the JA pathway (Pieterse et al. 2009; Zander et al. 2014). For example, increased H₂O₂ levels lead to the induction of ET biosynthesis (Chamnonngpol et al. 1998; Vandenabeele et al. 2003). As well, enhanced GSH levels in *NtGp* tobacco lines overexpressing the γ -GCS gene leads to the accumulation of 1-aminocyclopropane-1-carboxylate, the immediate precursor to ET (Chamnonngpol et al. 1998; Ghanta et al. 2014; Vandenabeele et al. 2003; Xu et al. 2008). Thus, H₂O₂ and GSH may lead to elevated ET levels that modulate JA-dependent defense responses. Binding of ET to a heterodimeric receptor formed through the interaction between ET resistant1 and ET response sensor1 on the endoplasmic reticulum inactivates the negative regulatory protein CTR1 (constitutive triple response 1) (Wang et al. 2002; Yoo et al. 2009; Zhao and Guo 2011). Upon CTR1 inactivation, the ET signal transduction cascade leads to the expression of the genes encoding ERF1 (ET response factor 1) and the ORA59 protein (octadecanoid-responsive *Arabidopsis* AP2/ERF) that regulate two branches of the ET-signaling pathway and integrate ET responses with the JA pathway (Lorenzo et al. 2003; Pré et al. 2008).

ET is implicated in the increased susceptibility of *Arabidopsis* or tomato to generalist caterpillars, such as *S. littoralis* and *Helicoverpa zea*, respectively (Stotz et al. 2000; Tian et al. 2014). In contrast, ET is correlated with plant resistance to *Spodoptera frugiperda* herbivory in maize (Harfouche et al. 2006). This variation in the role of ET in plant responses to caterpillar herbivory may reflect the plant or insect species or the timing of responses (early versus late). In particular, caterpillar oral

secretions activate ET-responsive pathways to antagonize the JA-dependent pathway (Diezel et al. 2009). However, some plant species prioritize the JA pathway to antagonize ET-dependent pathways making the plant less appealing to the herbivore (Verhage et al. 2011).

There are multiple putative signaling nodes between the JA and ET pathways mediated by the MYC and ERF1 or ORA59 branches, respectively, that may be exploited by caterpillar herbivores (Pieterse et al. 2012; Verhage et al. 2011; Zhu et al. 2011). Chewing herbivory generally activates the MYC branch of the JA pathway. However, ET signals from herbivore feeding are integrated into the ERF1 or ORA59 branches to activate or attenuate the MYC pathway (Koornneef and Pieterse 2008; Leon-Reyes et al. 2009; Lorenzo et al. 2004; Pieterse et al. 2012, 2013; Spoel et al. 2003). For example, ET has a positive effect on JA-dependent proteinase inhibitor activity in tomato but attenuates JA-induced nicotine biosynthesis in tobacco (Kahl et al. 2000; Winz and Baldwin 2001). Zander et al. (2014) have recently shown that activation of the SA pathway antagonizes the ORA59 branch of the ET pathway. Given that previous research suggests that caterpillar LS antagonizes the JA-dependent induced responses by activating the SA signaling pathway, the involvement of ET in modulating these pathways is of key importance.

The model legume *Medicago truncatula* Gaertn. was used to investigate the role of ET in caterpillar LS-mediated suppression of plant IR. The unique symbiotic relationship between legumes such as *M. truncatula* and nitrogen-fixing rhizobia may lead to hormonal interactions that are distinct from other plant species, such as *Arabidopsis* (Anderson and Singh 2011; Oldroyd and Downie 2008). In the early infection process, GSH and related molecules act as antioxidants, whereas later in nodule development, ASC and GSH levels are not linked with oxidative stress but, rather, cues for developmental programming such as nodule senescence (Pauly et al. 2006). In comparison, less is known about the role of these redox metabolites in response to foliar insect herbivory in legumes. Early responses of legume leaves to mechanical damage include H₂O₂ and cellular Ca²⁺ influxes (Arimura et al. 2008; Maffei et al. 2006). However, there are distinctions between wounding caused by mechanical damage and caterpillar feeding. In legumes such as lima bean, the zone of H₂O₂ deposited on wound sites is larger when caused by caterpillar herbivory (Maffei et al. 2006). Also, ET produced in response to *S. exigua* herbivory modulates early Ca²⁺ fluxes in *M. truncatula* (Arimura et al. 2008). Wounding of *M. truncatula*, either through mechanical damage or caterpillar herbivory, results in a strong JA burst leading to downstream defense responses that may be influenced by other phytohormone signaling pathways (Tretner et al. 2008; Weech et al. 2008). For example, terpenoid volatiles produced in response to caterpillar herbivory that act to attract natural enemies of the insect pest are synergistically increased by ET and JA (Arimura et al. 2008; Gomez et al. 2005).

Insect salivary effectors are increasingly shown to play a critical role in activating phytohormone pathways to shape the plant's final defense response. In fact, although one current view generalizes that plant defense mechanisms against phloem feeders such as aphids is primarily mediated through the methyl salicylate/SA pathways, this standpoint is being challenged by emerging evidence supporting the important role of JA-mediated defenses in plant resistance against aphids (Kamphuis et al. 2013). Transcriptional profiles comparing plant responses to aphid herbivory in resistant ('Jester') and susceptible ('A17') *M. truncatula* lines show similar expression levels of SA- and ET-responsive genes but elevated levels of JA-dependent transcripts in the resistant line (Gao et al. 2007). It is important to note that evidence also points to one or more effectors in aphid saliva that activate, either singly or together, SA and ET signaling

pathways to suppress the JA-resistance pathway (Jaouannet et al. 2014). In a similar fashion, caterpillar LS may modulate JA-mediated defense responses through activation of phytohormone pathways.

In this study, oxidative stress, hormone levels, and gene and protein markers of JA-dependent IR were compared in wild-type *M. truncatula* and the ET-insensitive *skl* mutant that has a mutation in MtSk11, an ortholog of AtEIN2 (Penmettsa and Cook 1997; Penmettsa et al. 2008). *M. truncatula* plants were infested by fourth instar caterpillars of *S. exigua*, a generalist pest species with high LS GOX activity (Merkx-Jacques and Bede 2005). To understand the potential role of LS in these interactions, plant responses to caterpillars with normal versus impaired labial salivary secretions (cauterized) were compared.

RESULTS AND DISCUSSION

Caterpillar LS affects *Medicago truncatula* cellular oxidative stress.

In both wild-type *M. truncatula* and the ET-insensitive *skl* mutant, total ASC does not change in the initial 45 min after

herbivory (Fig. 1A and B). As well, the ratio of oxidized-to-reduced ASC (DHA/ASC) does not change in control plants or in plants subject to herbivory by caterpillars with impaired labial salivary secretions over the 45-min time course. In comparison, herbivory by caterpillars with normal labial salivary secretions results in an increase in the cellular DHA/ASC ratio at 45 min (Fig. 1A). This LS-specific response is not seen in the *skl* mutant, indicating that caterpillar LS-associated changes in plant redox metabolites require ET signaling.

Total and reduced GSH levels do not change in response to caterpillar herbivory over the first 45 min in control and cauterized treatment in both the *M. truncatula* wild type and *skl* mutant plants (Fig. 1C and D). In response to herbivory by caterpillars with normal labial salivary secretions, a transient increase in GSSG levels and, therefore, in the GSSG/GSH ratio is observed in wounded leaves of wild-type *M. truncatula* within 15 to 30 min of the initiation of herbivory as compared with control plants (Fig. 1C). GSSG decreases to basal levels at 45 min. In contrast, GSH, GSSG, or total GSH levels do not change in the *skl* mutant in response to caterpillar feeding (Fig. 1D).

Fig. 1. Time course of redox metabolites in *Medicago truncatula* plants in response to *Spodoptera exigua* caterpillar herbivory. Six-week-old *M. truncatula* wildtype (+/+) or *skl* mutant plants were subjected to herbivory by fourth instar *S. exigua* caterpillars. To assess the involvement of caterpillar labial saliva (LS) in plant responses, two populations of caterpillars were used, i.e., those with intact (caterpillar) or impaired (cauterized) LS secretion. **A**, Ascorbate (ASC) levels in *M. truncatula* wild type plants or **B**, *skl* mutants. Solid bars represent reduced ASC and open bars represent oxidized ASC (DHA). At 45 min, the ratio of DHA/ASC is significantly increased in wild-type plants infested by caterpillars with intact LS secretions. **C**, Glutathione (GSH) levels in *M. truncatula* wild type plants and **D**, *skl* mutants. Solid bars represent reduced GSH and open bars represent oxidized glutathione (GSSG). In wild-type plants, GSSG and, thus, the ratio of GSSG/GSH is significantly increased in plants subject to herbivory by caterpillars with intact salivary secretions at 15 to 30 min after infestation as compared with control plants. Bars represent the means of five to eight independent biological replicates $\pm$ standard error. Within each genotype, redox metabolites were analyzed by two-way analysis of variance (ANOVA). When an interaction was detected (time $\times$ treatment), samples within each treatment were analyzed by one-way ANOVA, followed by Tukey honest significant difference post hoc test to identify the significant effect ($P \leq 0.05$). Significant differences are denoted by asterisks.

Activation of the ASC/GSH cycle in response to pathogen and herbivore attack is critical to maintain cellular redox status (Ball et al. 2004; Espunya et al. 2012; Parisy et al. 2007; Schlaeppli et al. 2008). In fact, *pad2-1* mutants that have lower GSH levels are more susceptible to caterpillar herbivory highlighting the important role of the ASC/GSH cycle in plant resistance (Parisy et al. 2007). Elevated total and GSH levels in response to biotrophic pathogen infection activate NPR1, an important modulator of the SAR pathway (Mateo et al. 2006; Mou et al. 2003). GSH may also be involved in regulating signal transduction pathways by affecting protein posttranslational modification (Han et al. 2013; Spoel and Loake 2011). In the current study and previously, we investigated early redox response of two model plants, *M. truncatula* and *A. thaliana*, to caterpillar herbivory. In *Arabidopsis*, *S. exigua* caterpillar LS helps maintain a reductive cellular environment, since total and GSH levels are observed in plants infested by caterpillars with impaired labial salivary secretions (Paudel et al. 2013). In contrast, even though the current study shows no effect on the total level of either ASC or GSH, the ratio of DHA/ASC, as well as the level of GSSG and, hence, the ratio of GSSG/GSH, increases in *M. truncatula* as an early response to caterpillar feeding. These caterpillar-specific (GSH) and LS-dependent (ASC) changes are not observed in the *skl* mutant, indicating that ET signaling is needed for the activation of the ASC/GSH cycle in response to caterpillar herbivory in *M. truncatula*.

The JA burst observed upon caterpillar herbivory is not affected by ET.

As expected, a JA burst is observed in response to caterpillar herbivory (Fig. 2A and B). Free JA and bioactive JA-Ile are strongly elevated in both genotypes of *M. truncatula*, wild type

and *skl* mutants, upon *S. exigua* caterpillar feeding (Fig. 2A and B). Therefore, in *M. truncatula*, ET does not mediate the early JA burst in response to caterpillar feeding. Also, a LS-specific difference in JA levels is not observed in *M. truncatula*, unlike previous observations in *Arabidopsis* (Lan et al. 2014; Weech et al. 2008). In *Arabidopsis*, caterpillar LS impairs the plant's ability to mount a full JA burst. In *M. truncatula*, the caterpillar-specific increase in 12-*oxo*-phytodienoic acid (OPDA), a precursor in the JA pathway and bioactive molecule, or SA are not observed (Fig. 2C; SA data not shown). Abscisic acid (ABA) levels are unaffected by caterpillar herbivory in wild-type plants but increase in *skl* mutants fed upon by caterpillars with intact labial salivary secretions, suggesting that LS-induced ET suppresses ABA levels (Fig. 2D). In *Arabidopsis*, ABA is involved in priming systemic leaves in response to herbivory by specialist caterpillars of the cabbage white butterfly *Pieris rapae* (Vos et al. 2013).

Markers of the JA, SA, and ET pathways are differentially expressed in response to caterpillar LS.

As expected from previous research (Darwish et al. 2008), *Rubisco activase* (*MtRCA*), *strictosidine synthase-like* (*MtSTR*-like), and *E3 ubiquitin-protein ligase* (*MtXBAT31*-like) expression is lower in response to caterpillar herbivory (Fig. 3A, B, and D); a LS-specific difference is not observed. In *Nicotiana attenuata*, RCA has been implicated in the redirection of defensive phytohormones, such as JA-Ile, into the less active methyl JA (Mitra and Baldwin 2014). Therefore, this observed downregulation of *MtRCA* expression in response to caterpillar herbivory may reflect the plant's strategy to avoid this redirection and activate plant defenses. Expression of the receptor protein kinase (*MtRPK*) gene is also suppressed in response to insect

Fig. 2. *Medicago truncatula* phytohormone levels in response to *Spodoptera exigua* caterpillar herbivory. Six-week-old *M. truncatula* wild type or *skl* mutant plants were subjected to herbivory by fourth instar *S. exigua* caterpillars for 10 h. To assess the involvement of caterpillar labial saliva (LS) in plant responses, two populations of caterpillars were used, i.e., those with intact (caterpillar, black bars) or impaired (caterpillar, hatched bars) LS secretions. **A**, Foliar levels of jasmonate (JA), **B**, jasmonyl-isoleucine (JA-Ile), **C**, 12-*oxo*-phytodienoic acid (OPDA), and **D**, abscisic acid (ABA) were compared. Bars represent the means of three independent biological replicates $\pm$ standard error. Hormone levels were analyzed by one-way analysis of variance within each genotype followed by a Tukey honest significant difference posthoc test. Letters indicate significant difference between treatments ($P \leq 0.05$).

feeding (Fig. 3C), contrary to what was observed by Darwish et al. (2008). This likely reflects differences between early (1 h) (Darwish et al. 2008) and later (10 h, this study) regulation of this gene. Caterpillar-dependent suppression of *MtSTR*-like, *MtRPK*, and *MtXBAT31*-like is alleviated in the ET-insensitive *skl* mutants. However, for *MtSTR*-like and *MtXBAT31*-like, this may reflect the already low basal constitutive levels.

Vegetative storage protein (MtVSP) and *hevein-like protein (MtHEL, PR4)* are markers of the JA and JA/ET pathways, respectively (Pré et al. 2008). In *Arabidopsis*, *AtHEL* expression integrates signals from both the JA and ET pathway via the transcription factor ORA59 (Pré et al. 2008). In comparison, *AtVSP1* expression is ORA59-independent. Similar to the JA profile (Fig. 2A and B), caterpillar feeding enhances *MtVSP* expression, confirming the involvement of the MYC branch of the JA-mediated defense pathway (Fig. 3E). A caterpillar LS-dependent regulation is not observed, in agreement with previous observation of MYC-dependent gene expression in *Arabidopsis* (Lan et al. 2014). A similar expression pattern in the ET-insensitive *skl* mutant was expected, since JA but not ET plays a central role in the induction of the *VSP* gene (Pré et al. 2008; Verhage et al. 2011). In contrast, *MtHEL* transcripts are suppressed in response to *S. exigua* herbivory in both plant genotypes (Fig. 3F). The suppression of *AtHEL* by the antagonistic SA-mediated pathway is independent of NPR1 (Ndamukong et al. 2007). Therefore, the observed suppression of *MtHEL* could be due to the antagonistic effect of the caterpillar-induced

MYC branch on the AP2/ERF branch of the JA pathway (Lorenzo et al. 2004; Pieterse et al. 2012). In contrast, the antagonism mediated by the MYC branch on *AtERF1* expression is not observed in *Arabidopsis* subject to *S. exigua* caterpillar herbivory or in tomato plants infested by *H. zea* caterpillars (Paudel et al. 2013; Tian et al. 2014). Therefore, this may represent the differential regulation of the ORA59 and ERF1 branches of the JA/ET pathway in different plant species in response to caterpillar herbivory (Verhage et al. 2011). Zander et al. (2014) have recently shown that SA mediates the inhibition of the ORA59 branch of the ET pathway through TGA transcription factors in *Arabidopsis*. However, if this was the case in *Medicago* spp., we might expect a caterpillar LS-dependent difference, which we did not observe. However, this may reflect species-related or timing differences, as suggested by Zander et al. (2014).

Pathogenesis-related protein 1 (MtPRI) is a marker of the NPR1-dependent SAR pathway (Pieterse et al. 2012; Pré et al. 2008). LS-specific induction of *MtPRI* is observed in response to caterpillar feeding. This suggests that one or more effectors in the caterpillar LS activates the SAR pathway (Fig. 3G). In *Arabidopsis*, the LS-specific induction of the *AtPRI* gene is also observed late (36 h) but not early (10 h) after the initiation of *S. exigua* herbivory (Lan et al. 2014; Paudel et al. 2013). *MtPRI* expression is ET-dependent (Fig. 3G), in agreement with previous observations that ET enhances the plant response to SA-dependent gene expression (Leon-Reyes et al. 2009).

Fig. 3. *Medicago truncatula* defense-related gene expression in response to *Spodoptera exigua* caterpillar herbivory. Six-week-old *M. truncatula* wildtype or *skl* mutant plants were subjected to herbivory by fourth instar *S. exigua* caterpillars for 10 h. To assess the involvement of caterpillar labial saliva (LS) in plant responses, two populations of caterpillars were used, i.e., those with intact (caterpillar, black bars) or impaired (cauterized, hatched bars) LS secretions. **A**, Relative expression of *MtRCA*, **B**, *MtSTR*-like, **C**, *MtRPK*, **D**, *MtXBAT31*-like, **E**, *MtVSP*, **F**, *MtHEL*, and **G**, *MtPRI* genes were compared in caterpillar-wounded or undamaged leaves from control plants. Expression levels were normalized to the constitutively expressed *MtGADPH* gene. Bars represent the means of four to five independent biological replicates ± standard error. Transcript expression levels were analyzed by one-way analysis of variance within each genotype followed by a Tukey honest significant difference posthoc test. Letters indicate significant difference between treatments ($P \leq 0.05$).

Caterpillar LS-dependent suppression of TI activity.

In wild-type *M. truncatula*, TI is significantly higher in plants infested by caterpillars with impaired labial salivary secretions than in plants attacked by normal caterpillars or control plants, suggesting that one or more effectors in *S. exigua* caterpillar LS prevents the plant from mounting a full JA defense response (Fig. 4A). The LS-specific suppression of TI is abolished in the ET-insensitive *skl* mutant. The activity of the defense-related protein PPO is not affected by caterpillar herbivory in either genotype (Fig. 4B).

Conclusions.

In addition to rapid cellular Ca^{2+} influx and changes in membrane potential, one of the first plant responses to caterpillar herbivory is changes to cellular redox balance (Maffei et al. 2006). This may manifest itself by increases in total ASC or GSH levels or shifts in the balance of oxidized-to-reduced metabolites (Noctor et al. 2012). In *M. truncatula*, total ASC and GSH levels do not change in response to caterpillar herbivory. Instead, changes in the DHA/ASC and GSH ratios, indicative of oxidative stress, are observed within the first 45 min after caterpillar herbivory (Fig. 1A and C). With ASC, the LS-specific increase in ASC ratio is not observed in the *skl* mutant, suggesting that this caterpillar LS-dependent difference requires ET signaling (Fig. 1B). When compared with control plants, plants infested by caterpillars with intact labial salivary secretions have a transient increase in GSSG and also, therefore, the GSSG/GSH ratio within the first half hour after attack (Fig. 1C). This change in oxidative stress is not observed in the *skl* mutant (Fig. 1D). This is of interest since, in *Arabidopsis*, GSSG levels are positively correlated with JA defense responses and points to the role of ET in the regulation of cellular redox changes (Mhamdi et al. 2010). Therefore, in *M. truncatula*, caterpillar herbivory results in an increase in oxidative stress within the first hour. This contrasts with *Arabidopsis*, in which caterpillar LS suppresses changes in oxidative stress seen in plants subject to attack by caterpillars with impaired labial salivary secretions (Paudel et al. 2013). These apparently contradictory strategies may reflect differences in plant defense responses, since, in *Arabidopsis*, GSH is a precursor to key defensive compounds, i.e., glucosinolates (Halkier and Gershenzon 2006), or may reflect the influence of host plant nutritional quality (i.e., *Arabidopsis* versus *M. truncatula*) on one or more effector levels in the caterpillar LS (Afshar et al. 2010; Merks-Jacques and Bede 2005; J. C. Bede unpublished data).

Later plant responses to caterpillar herbivory involve changes in phytohormones, gene expression, and defense-related proteins. Ten hours after the initiation of herbivory, a strong JA burst is observed. Unlike *Arabidopsis*, LS-specific differences in JA phytohormones, i.e., jasmonic acid or JA-Ile, are not observed (Fig. 2A and B) (Lan et al. 2014). In contrast, similar to previous studies in *Arabidopsis*, caterpillar LS-related differences in SA hormone levels are not observed (Lan et al. 2014; Weech et al. 2008).

Caterpillar herbivory results in the suppression of *MtRCA*, *MtSTR*-like, and *MtXBAT31*-like gene expression (Darwish et al. 2008). *MtVSP*, a marker of the JA pathway, is induced in response to caterpillar herbivory in a LS- and ET-independent manner (Fig. 3E). In contrast, levels of *MtHEL*, a marker of the JA/ET pathway, is lower in response to caterpillar herbivory (Fig. 3F). Since this pattern of expression is also LS- and ET-independent, this may reflect the involvement of other antagonistic phytohormone pathways, such as the gibberellin/delta pathway, in these plant-insect interactions. Of key interest is the expression pattern of the SA marker gene *MtPR1*. As in *Arabidopsis*, caterpillar LS-dependent *PR1* expression is observed in *M. truncatula* (Fig. 1G) (Paudel et al. 2013). This is alleviated in the *skl* mutant, illustrating the role of ET in the positive enhancement of SA-dependent *MtPR1* gene expression, as has been previously shown in *Arabidopsis* (Leon-Reyes et al. 2009). This result indicates that, in response to caterpillar herbivory, both SA and ET pathways are activated in addition to the expected JA signaling pathway. Finally, caterpillar LS suppresses the induction of the important defensive protein TI in an ET-dependent manner. Together, these results support the model that caterpillar LS activates the SA/NPR1 pathway to interfere with plant induced resistance and points to the importance of ET in modulating this crosstalk (Fig. 5).

MATERIALS AND METHODS

Chemicals.

All chemicals were purchased from Sigma Chemical Co., unless otherwise noted.

Plant growth conditions.

M. truncatula cv. Jemalong A17 seeds were scarified in concentrated sulfuric acid (12 min) and were then thoroughly washed with sterile distilled water. Two genotypes were

Fig. 4. *Medicago truncatula* defense-related protein levels and activity in response to *Spodoptera exigua* caterpillar herbivory. Six-week-old *M. truncatula* wild type or *skl* mutant plants were subjected to herbivory by fourth instar *S. exigua* caterpillars for 34 h. To assess the involvement of caterpillar labial saliva (LS) in plant responses, two populations of caterpillars were used, i.e., those with intact (caterpillar, black bars) or impaired (cauterized, hatched bars) LS secretions. **A**, Trypsin inhibitor (TI) levels and **B**, polyphenol oxidase (PPO) activity were compared in caterpillar-wounded or undamaged leaves from control plants. Bars represent the means of 10 to 12 independent biological replicates $\pm$ standard error. TI levels and PPO activity were analyzed by one-way analysis of variance within each genotype, followed by a Tukey honest significant difference posthoc test. Letters indicate significant difference between treatments ($P \leq 0.05$).

planted; wild type and the ET-insensitive *skl* mutant. After stratification at 4°C for 2 days followed by 2 days at room temperature, seedlings were sown in pasteurized Agro mix (Fafard). Plants were kept in growth cabinets (light intensity 260 $\mu\text{Em}^{-2}\text{s}^{-1}$, 16 h of light and 8 h of dark, 22°C) and were watered three times weekly with dilute 20:20:20 NPK fertilizer (1.05 g of NPK per 7 liters H_2O). At three weeks, seedlings were transplanted to 1-liter pots.

Caterpillar maintenance conditions.

Caterpillars of the beet armyworm *S. exigua* were reared on a wheat germ-based artificial diet (Bio-Serv). The colony was maintained in growth cabinets (28°C, 60% relative humidity, 16 h of light and 8 h of dark). Fourth instar caterpillars were used in the experiments. Caterpillars were fed on *M. truncatula* cv. Jemalong A17 (wild-type “feeder” plants) for 24 h before being transferred to experimental plants.

Ablation of the caterpillar spinneret (cauterization).

To evaluate the role of caterpillar LS in the modulation of plant IR, two populations of caterpillars were used; one with normal (mock) and the other with impaired (cauterized) labial salivary secretions. In the cauterized caterpillars, the spinneret was burned shut with a hot probe to prevent the secretion of LS (Musser et al. 2006). Since GOX is a key enzyme in caterpillar LS, a horseradish peroxidase (Hrp)-*o*-diaminobenzidine (DAB)-coupled reaction that detects GOX activity was performed to evaluate the success of the cauterization. Caterpillars, both mock and cauterized, fed on glass fiber discs saturated with a glucose/sucrose solution (1:1; 50 mg/ml each). After visible feeding, DAB tetrahydrochloride and Hrp (2.5 U) were added to the disc. The presence of GOX was indicated by a brown precipitation.

Experimental design.

Five- to six-week-old *M. truncatula* (wild type or *skl* mutant) plants received one of three treatments, either caterpillars with normal salivary secretions, cauterized caterpillars, or plants remained untouched (control). Treatments were performed at night (i.e., 1 h after lights were shut off in the growth cabinets), since these caterpillars exhibit a predominantly nocturnal feeding behavior. For herbivore treatments, three fourth-instar caterpillars were introduced per plant. To contain the caterpillars, a modified transparent bottle was fitted to the pot for all plants 1 week before the experiment, with netting used to cover the open tops. For the redox metabolite analysis, leaf samples were collected at 15-min intervals within the first 45 min following herbivory. For hormone analysis, whole plants cut at the crown were obtained after 10 h of herbivory. Caterpillar-fed leaves were collected for gene expression and defense protein analysis at 10 and 34 h after herbivory, respectively. Samples were immediately frozen in liquid nitrogen and were stored at -80°C until analysis. For ASC and GSH analysis, samples were collected from three independent plants and the experiment was repeated three times to generate nine biological replicates. Samples were collected from two to three independent plants and the experiment was repeated three to five times to generate at least ten biological replicates for analysis of phytohormones and defensive proteins or five biological replicates for gene expression studies.

Analysis of redox metabolites ASC and GSH.

ASC and GSH content of the leaf samples was analyzed following the protocol described by Queval and Noctor (2007). Frozen leaf samples were ground in liquid nitrogen and were extracted in acidic medium (0.2 N HCl), followed by neutralization with 0.2 N NaOH. Spectrophotometric analyses of total,

oxidized, and reduced forms of ASC and GSH were performed in a 96-well plate format, using the Tecan Infinite M200 Pro microplate reader as described below.

ASC.

In triplicate, samples were measured at 265 nm to determine levels of reduced ASC. Samples were then incubated with ASC oxidase (0.2 U) in 0.1 M sodium phosphate buffer (NaH_2PO_4), pH 5.6, for 8 min at room temperature. The difference in absorbance at 265 nm was used to calculate the level of reduced ASC in the sample (Queval and Noctor 2007). To determine total ASC levels, samples were incubated with 1 mM dithiothreitol (DTT) in 67.2 nM sodium phosphate buffer, pH 7.5, for 30 min at room temperature. DTT reduces DHA to ASC. An ASC standard curve (ranging from 40 to 240 μM) and blanks were included in each plate.

GSH.

GSH content was measured by the recycling assay method described by Rahman et al. (2006) and Queval and Noctor (2007). For total GSH (GSH + GSSG), sample extracts were incubated with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; 0.6 mM) in 0.1 M sodium phosphate buffer, pH 7.5. Glutathione reductase (0.015 U) and NADPH (0.75 mM) converts GSSG to GSH. GSH reacts with DTNB to produce GS-TNB, which then converts into TNB (5-thio-2-nitrobenzoic acid), which is measured at 412 nm. TNB levels were measured as absorbance at 412 nm at 5-s intervals for 2 min. The slope of the first 90 s was used to calculate GSH concentration, using a standard curve of free GSH (ranging from 1 to 50 μM).

Fig. 5. Model of the role of ethylene (ET) in the caterpillar labial saliva (LS)-mediated modulation of jasmonate (JA)-dependent defense responses in *Medicago truncatula*. **A**, In response to *Spodoptera exigua* caterpillar herbivory, a JA burst is observed, leading to the induction of *MtVSP* and suppression of *MtHel* expression. **B**, Caterpillar LS modulates plant responses. Rapidly after the onset of herbivory, a LS-dependent increase in oxidized-to-reduced ascorbate is observed. Also, an increase in the oxidized glutathione/glutathione (GSSG/GSH) ratio is observed in response to herbivory by caterpillars with intact LS secretions as compared with control plants. LS-dependent induction of the systemic acquired resistance marker *MtPRI* and the suppression of the defensive protein trypsin inhibitor is observed. These differences are alleviated in the *skl* mutant indicating that ET signaling plays a role in the caterpillar LS-mediated modulation of plant defense responses.

To measure GSSG, the neutralized sample was incubated with 1 μ l of 2-vinylridine (VPD) for 30 min at room temperature. VPD precipitates GSH, which is then removed by centrifugation at $15,700 \times g$ for 5 min. The VPD-treated extract was diluted with 0.1 M sodium phosphate buffer, pH 7.5, and the concentration of GSSG was measured by the recycling assay, as described above. A standard curve generated using free GSSG (ranging from 0.1 to 2.5 μ M) was used to calculate GSSG concentration. Reduced GSH in the sample was calculated by subtracting $2 \times$ GSSG from the total GSH. Samples were analyzed in triplicate, and negative controls were included in every plate.

Hormone analysis.

JA, JA-Ile, OPDA, SA, and ABA levels were analyzed in the Proteomics and Mass Spectrometry facility at the Danforth Plant Science Center (St. Louis, MO, U.S.A.) by liquid chromatography coupled to tandem mass spectrometry. Samples were ground, using a TissueLyser for 2 min at a frequency of 15 Hz s^{-1} . Hormones were extracted from lyophilized leaf samples in ice cold methanol (MeOH)/acetonitrile (ACN) (1:1, vol/vol). Samples were spiked with deuterium-labeled internal standards of SA (D^5 -SA), ABA (D^6 -ABA), and JA (D^2 -JA). Supernatants were collected after centrifugation at $16,000 \times g$ and extraction of pellets was repeated. Pooled supernatants were evaporated using a speedvac, before redissolving the pellets in 200 μ l of 30% MeOH for hormone analysis. Chromatographic separation of metabolites was accomplished on a monolithic C_{18} column (Onyx, $4.6 \times 100 \text{ mm}$; Phenomenex), using a binary solvent of 0.1% acetic acid in high-performance liquid chromatography-grade water (vol/vol) (solvent A) and 90% ACN with 0.1% acetic acid (vol/vol) (solvent B) with a flow rate of 1 ml min^{-1} . A 4000-QTRAP (AB Sciex) was used to acquire mass spectrometry spectra. Parameters for analysis were set as follows: electron spray ionization in the negative mode (TurboIonSpray), capillary voltage $-4,500$, nebulizer gas (N_2) 50 arbitrary units (a.u.), heater gas 50 a.u., curtain gas 25 a.u., collision activation dissociation high, temperature 550°C . Compounds were detected using multiple reaction monitoring transitions that were optimized for each phytohormone and deuterium-labeled standards (Pan et al. 2010). Concentrations were determined from the standard curves of known phytohormones.

Gene expression analysis.

RNA extraction and reverse transcription. Total RNA was extracted from samples using the RNeasy kit (Qiagen), following the manufacturer's instructions. After determining RNA concentration and quality (260/280 of approximately 2), cDNA was generated using a QuantiTect reverse transcription kit (Qiagen). Before reverse transcription, genomic DNA in the samples was degraded using the DNase included in the kit. The absence of genomic DNA contamination was confirmed using primers (5'-CTCTCCTGCATTTCCTCACTTTC-3' and 5'-TTCTTGACCC TACCAACATCA-3') that amplify an introgenic region by polymerase chain reaction (PCR) (Darwish et al. 2008). As a positive control, genomic DNA was used as template. cDNA samples were diluted 1:10 and transcript abundance was measured by quantitative real time (qRT)-PCR.

qRT-PCR. Gene expression was analyzed by SYBR green-based qRT-PCR, using a MX3000p thermo-cycler (Stratagene). Reactions contained $1 \times$ SYBR Green with low ROX (Absolute Blue; Thermo Scientific), cDNA, and $1 \mu\text{M}$ of both forward and reverse primers. Thermocycler conditions were as follows: 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing for 30 s (55 to 60°C for the different genes of interest [GOI]), and elongation at 70°C for 30 s. Primers for the GOI

and reference gene *MtGADPH* are listed in Supplementary Table 1. Five biological replicates ($n = 5$) for each treatment from the two genotypes were analyzed in an opaque white 96-well plate. Samples were spotted in duplicate, and nontemplate controls were included in each plate. Two technical plate replicates were performed.

The relative expression ratio of the target gene versus the reference gene was calculated as $R0_{GOI}/R0_{REF}$, where $R0$ is the initial template concentration in each reaction. $R0$ was calculated using the formula $R0 = 1/(1+E)^{Ct}$, where E is the average efficiency of gene in the exponential phase and Ct is the threshold cycle (Zhao and Fernald 2005).

Analysis of defensive proteins. TI levels and PPO activity were measured in the leaf extracts following protocols described by Weech et al. (2008). For soluble protein extraction, ice-cold extraction buffer (0.1 M sodium phosphate buffer, NaH_2PO_4 , pH 7, containing 0.1% triton-X and 7% polyvinylpyrrolidone) was added to finely ground leaf samples. After centrifugation at $15,700 \times g$ for 10 min., the supernatant was transferred to a fresh tube. For the PPO assay, proteinase inhibitor cocktail (0.5 $\times$) was added to extraction buffer to inhibit protein proteolysis.

The Bradford assay was performed to calculate the total soluble protein in the sample. A standard curve was generated, using bovine serum albumin ranging from 5 to 100 μg . Standards and samples were incubated with Bradford reagent (Fisher Scientific) for 10 min, followed by A_{590} measurement of absorbance at 590 nm (Bradford 1976).

TI assay. Leaf soluble protein extracts were incubated with bovine trypsin (0.5 μg) at 37°C for 20 min with constant shaking. The trypsin substrate *N*-benzoyl-L-arginyl- β -naphthylamide hydrochloride (BANA; 2.6 mM) was added and samples were incubated for an additional 80 min, with constant shaking. Trypsin cleaves BANA to release β -naphthalene. After the reaction was stopped by adding HCl (0.47%), *p*-dimethyl-amino-cinnamaldehyde (0.025%), which reacts with β -naphthalene, was added and the absorbance was read at 540 nm. Samples were analyzed in triplicate. Also, a standard curve of soybean TI, type 1S (ranging from 1 ng to 5 μg), negative controls, and blanks were included in each plate.

PPO assay. Dimethyl formamide (0.1%) and 3-methyl-2-benzothiazolinone hydrazine (0.2 mM) were added to triplicate soluble protein extracts. These compounds stabilize the reactive quinones that are produced by PPO. After the addition of substrate dopamine hydrochloride (35 mM), reaction kinetics was measured at 476 nm every 10 s for 3 min. The rate for the first 90 s was used for analysis. In addition, sodium dodecyl sulfate-treated and boiled samples (negative controls), tyrosinase (positive control), and blanks were included in each plate.

Statistical analysis.

A two-way analysis of variance (ANOVA) was used to analyze redox metabolites using SPSS (version 20) ($P \leq 0.05$) (Supplementary Table 2). Since ET may affect plant growth and development, genotypes were analyzed separately (Benavente and Alonso 2006). When an interaction was detected (i.e., time $\times$ treatment), samples within each treatment were analyzed by one-way ANOVA, followed by a Tukey honest significant difference (HSD) posthoc test to identify the significant effect.

Gene expression, hormone levels, TI levels, and PPO activity were analyzed by one-way ANOVA within each genotype, followed by a Tukey HSD posthoc test to determine statistical differences ($P \leq 0.05$) between treatments (Supplementary Table 3) (Rieu and Powers 2009).

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