Caterpillar herbivory and salivary enzymes decrease transcript levels of *Medicago truncatula* genes encoding early enzymes in terpenoid biosynthesis[*]

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Abstract

In response to caterpillar herbivory, alfalfa and related plant species defend themselves through the induction of saponin and volatile terpenoid biosynthesis. Both these types of defensive compounds are derived from the metabolic intermediate, isopentenyl diphosphate (IPP). In plants, two distinct biosynthetic pathways can generate IPP; the cytosolic mevalonate pathway and the plastid-associated 2C-methyl erythritol 4-phosphate (MEP) pathway. In *Medicago truncatula*, transcript levels of key regulatory genes active in the early steps of these biosynthetic pathways were measured in response to larval herbivory by the beet armyworm, *Spodoptera exigua*. Transcripts encoding enzymes at early steps of both terpenoid pathways were lower in caterpillar-damaged leaves. Higher degrees of herbivore damage accentuated the decrease in transcript levels; however, transcript amounts were not affected by insect larval stage. Insect larvae, manipulated to reduce labial gland salivary secretions, were used to examine the role of the salivary elicitors in modulating gene expression. Results suggest that an insect salivary factor, possibly glucose oxidase (GOX), may be involved in reduction of transcript levels following herbivory. Addition of GOX or hydrogen peroxide to mechanically wounded leaves confirm these findings. In comparison, transcript levels of a gene encoding a putative terpene synthase are induced in mechanically- or insect-damaged leaves. These data show that insect salivary factors can act to suppress transcript levels of genes involved in plant defense pathways. Findings also suggest that in response to stress such as insect herbivory, regulation occurs at the early steps of the MEP pathway.

Abbreviations: DMNT, (*3E*)-4,8-dimethyl-1,3,7-nonatriene; *dxs1*, D-1-deoxyxylulose 5-phosphate synthase; *dxr1*, D-1-deoxyxylulose 5-phosphate reductoisomerase; GOX, glucose oxidase; *hmg1*, 3-hydroxy-3-methylglutaryl CoA reductase; H_2O_2, hydrogen peroxide; IPP, isopentenyl diphosphate; MVA, mevalonate; MEP, 2C-methyl erythritol 4-phosphate; TMTT, (*E,E*)-4,8–12-trimethyl-1,3,7-tridecatetraene; *tps1*, terpene synthase

Introduction

Alfalfa (*Medicago sativa*) and related plants such as the barrel medic, *M. truncatula*, defend themselves against caterpillar herbivory by direct and indirect induced defenses (Small, 1996; Agrell *et al.*, 2003, 2004). Leaf glycosylated triterpenoids (saponins) are believed to protect the plant as direct defenses against caterpillar herbivory, primarily by interfering with absorption of dietary nutrients in the gut (Tava and Odoardi, 1997). One week after damage by caterpillars of the Egyptian cotton leafworm, *Spodoptera littoralis*, alfalfa foliar saponin levels increased (Agrell *et al.*, 2003, 2004); in particular, levels of medicagenic acid bidesmoside (3GlcA,28AraRhaXyl medicagenic acid) and soyasaponin I (3GlcAGalRha soyasapogenol B) doubled. In feeding studies, the aglycone of these saponins, medicagenic acid and soyasapogenol B, respectively, negatively impact caterpillar mortality, development and fecundity (Adel *et al.*, 2001).

An indirect defensive strategy of plants against insect herbivory is the synthesis and release of terpenoid and indole volatiles that are attractive to parasitoids or predators of the phytophagous insect (Paré and Tumlinson, 1999; Dicke and van Loop, 2000; Kessler and Baldwin, 2002). Qualitative and quantitative differences in volatile profiles show that herbivores elicit species-specific responses

(DeMoraes *et al.*, 1998). In addition, volatile semiochemicals may act as signals to neighbouring plants stimulating induction of defense responses (Arimura *et al.*, 2000; Karban *et al.*, 2000; Pickett and Poppy, 2001; Engelberth *et al.*, 2004). In response to *S. littoralis* caterpillar herbivory, a blend of volatiles is released from *M. truncatula* plants, of which the terpenoids β-caryophyllene, α-copaene, (+)-cyclosativene, (*3E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E,E*)-4,8-12-trimethyl-1,3,7-tridecatetraene (TMTT) appear to be signature volatiles for caterpillar herbivory (Koch *et al.*, 1999; Ozawa *et al.*, 2000; Leitner *et al.*, 2005). In *M. tuncatula*, as in other plant species, there are clear qualitative differences in volatile profiles depending on whether the herbivore utilizes a chewing or piercing-sucking feeding style (Leitner *et al.*, 2005).

In plants, the biosynthetic precursor of terpenoids and saponins, isopentenyl diphosphate (IPP), can be synthesized through either of two pathways; the mevalonate (MVA) pathway or the 2*C*-methyl erythritol 4-phosphate (MEP) pathway (Figure 1) (Lichtenthaler, 1999; Rohmer, 1999; Eichenrich *et al.*, 2001; Rodríguez-Concepción and Boronat, 2002). Labeling studies and measurement of natural isotope ratios indicate that lima bean plants exposed to insect herbivory synthesize the sesquiterpenoid derivative DMNT through the cytosolic MVA pathway and the
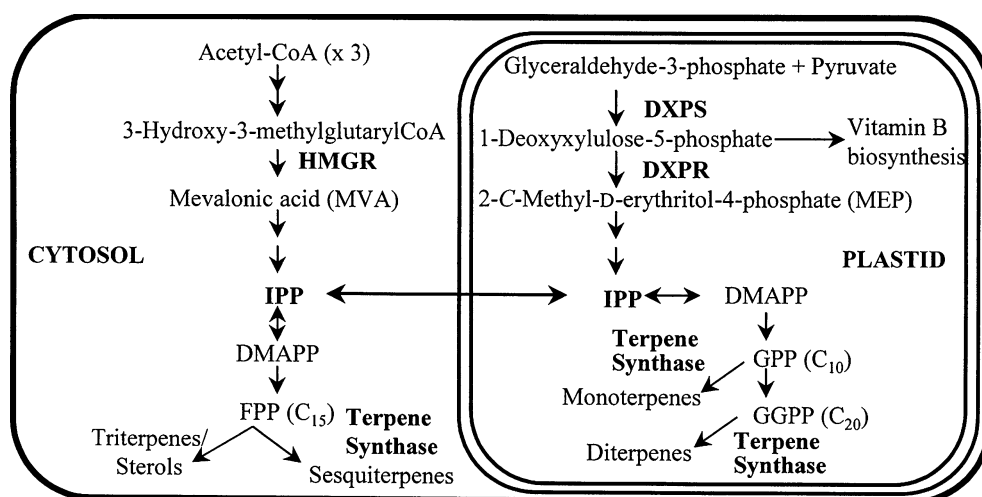

Figure 1. Cytosolic mevalonate and plastid 2*C*-methyl erythritol 4-phosphate terpenoid biosynthetic pathways leading to the production of volatile compounds, which are synthesized *de novo* and released upon insect herbivory, and saponins. Abbreviations: DMAPP: dimethyl allyl diphosphate; DXPR: D-1-deoxyxylulose 5-phosphate reductoisomerase; DXPS: D-1-deoxyxylulose 5-phosphate synthase; FPP: farnesyl diphosphate; GPP: geranyl diphosphate; GGPP: geranyl geranyl diphosphate; HMGR: 3-hydroxy-3-methylglutaryl CoA reductase; IPP: isopentenyl diphosphate; TPS1: terpene synthase.

monoterpene (E)-β-ocimene and diterpene derivative TMTT through the plastid-associated MEP pathway (Piel *et al.*, 1998; Jux *et al.*, 2001). However, if the MVA pathway is inhibited with cervistatin, DMNT is biosynthesized via the MEP pathway. Therefore, limited unidirectional exchange of isoprene units, such as IPP and geranyl diphosphate, occurs between the plastid and cytosol (Heintze *et al.*, 1990; McCaskill and Croteau, 1995; Bick and Lange, 2003). Little is known about the regulation of the MEP pathway or the possible cooperation between these two pathways in response to biotic stress, such as caterpillar herbivory. However, analysis of transcripts encoding biosynthetic enzymes in both the MVA- and MEP-terpenoid pathways in hybrid poplar trees (*Populus trichocarpa*×*deltoides*) showed that insect herbivory leads to differential regulation of genes in each of the pathways (Arimura *et al.*, 2004a).

Plant responses to insect herbivory have traditionally been viewed as an extension of the wound response. However, it is clear that although there are shared components with wounding, insect herbivores elicit unique responses in plants. In tobacco, cotton and corn, caterpillar herbivory produces distinct volatile profiles that allow female parasitic wasps to distinguish between damage by different herbivorous species (Turlings and Tumlinson, 1992; Röse *et al.*, 1996; Paré and Tumlinson, 1997; Kessler and Baldwin, 2001). For example, the specialist wasp, *Cardiochiles nigriceps*, can distinguish between tobacco seedlings damaged by caterpillars of the tobacco budworm, *Heliothis virescens*, or the corn earworm, *Helicoverpa zea*, and visit plants damaged by the preferred host, *H. virescens*, with greater frequency (DeMoares *et al.*, 1998). Therefore, there must be herbivore-specific signals, such as elicitors in the insect oral secretions which are recognized by the plant (Felton and Eichenseer, 1999). In chewing insects, such as caterpillars, solid food is ground externally in the preoral cavity, a pocket formed by insect mouthparts, the anterior and posterior labium and the lateral mandible and maxillae. Saliva is secreted from the mandibular or labial salivary glands and used to solubilize and transport food. Saliva or regurgitant may also contain enzymes that predigest plant material. Therefore, there is direct contact between plant tissues and insect oral secretions, which is comprised of regurgitant and mandibular and labial saliva.

Volicitin, [N-(17 hydroxylinolenoyl)-L-glutamine], a potent caterpillar elicitor involved in stimulating changes in plant volatile biosynthesis, was first isolated from larval *S. exigua* regurgitant (Alborn *et al.*, 1997). The volatile profile emitted from maize seedlings incubated in volicitin solution parallels that produced in response to caterpillar herbivory (Alborn *et al.*, 1997, 2000). Similarities in structure and induced gene expression patterns suggest that volicitin and jasmonic acid (JA), a key phytohormone in the mediation of wound stress responses, might elicit their physiological effects through similar mechanisms (Tumlinson *et al.*, 1999).

Lepidopteran larval saliva contains another elicitor, glucose oxidase (GOX), an enzyme that generates hydrogen peroxide (H_2O_2) through the oxidation of D-glucose (Felton and Eichenseer, 1999; Eichenseer *et al.*, 1999). H_2O_2, a component in the oxidative burst often associated with a pathogen-induced hypersensitive response, may stimulate salicylic acid (SA) accumulation (León *et al.*, 1995; Vandenabeele *et al.*, 2003). Plants with increased SA levels are often more vulnerable to insect herbivory (Bostock, 1999; Felton *et al.*, 1999; Preston *et al.*, 1999; Cipollini *et al.*, 2004). The biochemical basis for this response has been proposed to be an SA-mediated inhibition of JA biosynthesis or interference with JA-dependent gene expression (Doares *et al.*, 1995; Harms *et al.*, 1998; Gupta *et al.*, 2000; Spoel *et al.*, 2003). Insect-derived elicitors such as GOX might impact plant signaling dynamics via antagonism between JA- and SA-pathways (Bostock, 1999; Felton and Korth, 2000). However, in tobacco (*Nicotiana tabacum*), GOX suppression of the defensive secondary metabolite nicotine is not mediated by SA, suggesting that GOX functions at another level (Musser *et al.*, 2005).

In Lepidopteran larvae, salivary GOX activity originates predominantly in the labial glands, with less than 11% of total enzyme activity detected in mandibular glands (Eichenseer *et al.*, 1999). Therefore, cauterization of the spinneret, the specialized spine-like structure through which labial gland saliva is released, results in a dramatic reduction of GOX secretion. Induced plant defenses, measured in terms of insect performance, are significantly reduced on tobacco plants subjected to herbivory by insects with cauterized spinnerets as compared to normal insects. *H. zea*

neonates fed leaves from undamaged tobacco plants, leaves damaged by caterpillar herbivory, or leaves treated with purified salivary GOX showed increased body weight and survivorship compared to those insects that feed on tissue from plants damaged by insects with cauterized spinnerets (Musser et al., 2002).

The legume family Fabaceae represents one of the most significant agricultural taxa, not only in terms of the use of these plants in human and animal diets, but also because of their contribution to global nitrogen fixation through their symbiotic relationship with Sinorhizobium. Recently, M. truncatula, the barrel medic, has become a model for studying biological questions pertinent to this family (Oldroyd and Guerts, 2001; Zhu et al., 2005). We investigated the responses of M. truncatula to chewing herbivory by the generalist caterpillar, S. exigua. The influence of larval stages and infestation levels on accumulation of transcripts encoding enzymes in both terpenoid pathways was tested. To understand the role of salivary factors in the regulation of gene expression, caterpillar spinnerets were cauterized to inhibit labial salivary secretions from contacting insect-damaged leaves.

Materials and methods

Plant and insect maintenance

Medicago truncatula (A17) plants were grown in individual pots in a growth chamber (16 h light, 24 °C). Plants were watered three times a week and fertilized biweekly (15–30–15). Approximately 24 h prior to an experiment, 6-week-old plants were acclimated to greenhouse conditions.

Spodoptera exigua obtained as eggs from the USDA (Gast Rearing Laboratory, Starkville, MS) were reared on wheat germ base artificial diet (BioServ). Caterpillars were sorted based on instar and allowed to feed on M. truncatula plants. Insects were starved for approximately 10 h prior to the experiment.

Plant wounding and herbivory experiment

Plants were organized into groups of five and placed in 57 l containers. This prevented volatile contamination from other treatments but allowed neighbouring plants within an experimental group to be exposed to conspecific semiochemicals. One group was mechanically wounded by cutting approximately 1/3 of the middle leaf of a trifoliate at the tip with scissors, avoiding the midvein and removing between 5–10% of the total plant leaf tissue. In short term experiments (ST), three larvae from each instar (3rd or 5th) were placed on a plant, allowed to feed for 1 h and then removed. Approximately 5–10% of plant leaf tissue was removed. In long-term studies (LT), five 5th instar larvae were placed on each plant and allowed to feed for 6 h ingesting approximately 40% of total plant leaf tissue. All experiments began at 8 am and continued until 2 pm at which time pooled leaf material from each treatment group was divided into local (L, damaged) or systemic (S, leaves from undamaged trifoliates), frozen in liquid nitrogen and stored at −70 °C until use. All experiments were performed at least twice.

Spinneret cauterization

Insects (4th or 5th instar) were cooled on ice and a hot probe was applied to the spinneret of the immobilized caterpillar. The success of cauterization was monitored using the 3,3′ diaminobenzidine tetrahydrochloride (DAB) assay. A solution of glucose and sucrose (7.5 mg each) was added to glass filter disks (Whatman) and caterpillars were allowed to feed until approximately 10–20% of the disk was eaten. Disks were removed and assayed for GOX activity by addition of horseradish peroxidase (Sigma, 2.5 U in 50 mM sodium phosphate buffer, pH 7.0) and DAB (Pierce, 150 μg in an aqueous solution, pH 5.8). The presence of GOX was indicated by the production of a black-brown precipitate, this allowed differentiation between insects with normal (mock) or cauterized spinnerets. During the timeframe of these experiments, spinneret salivary secretion remained low even if moulting to the next larval instar occurred. Herbivory experiments using insects with cauterized and intact spinnerets were conducted following the protocol outlined above. There was no visible difference in the amount of plant material ingested by mock-treated larvae or larvae with cauterized spinnerets.

Glucose oxidase and hydrogen peroxide treatments

The middle leaf of a *M. truncatula* trifoliate was wounded with a tracing wheel. To each cut, 10 μl of sodium phosphate buffer, pH 7.0, GOX (Sigma G-6766, 0.0015 U) or hydrogen peroxide (Sigma, 22.5 nM) was added. The commercial source of fungal GOX used might also contain trace amounts of catalase, galactose oxidase, or other enzymes. The amount of GOX added to each leaf was selected to approximate one-half the labial gland GOX activity from *S. exigua* (0.0032 ± 0.001 U). Ten leaves were treated per plant and damaged (L) and systemic (S) leaves were collected 6 h after treatment.

RNA extraction and RNA blot analysis

Plant material was ground in liquid nitrogen and RNA was extracted using TriReagent according to the manufacturer's protocol for plant tissues (Molecular Research Center Inc., Cincinnati, OH). Fifteen microgram total RNA was electrophoresed on 1% (w/v) agarose formaldehyde gels and transferred to nylon membranes as described (Sambrook *et al.*, 1989). Probe DNA was prepared by standard polymerase chain reaction (PCR) amplification of insert DNA from selected *M. truncatula* expressed sequence tag (EST) clones. EST clones were identified from the *M. truncatula* database (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago) using a BLAST search (Altschul *et al.*, 1997). EST sequences or tentative consensus sequences were aligned against documented genes and similarities calculated. Protein similarity was defined as the number of identical and conserved amino acid residues in two sequences. Probes were radioisotope-labeled using a random primer method and hybridized under high stringency conditions (Church and Gilbert, 1984). Signals were detected by autoradiography and quantified using ImageQuant 5.1 software (Molecular Dynamics). Differences in transcript levels between plant treatments were analyzed by comparing signal intensities, with all values normalized to the value of the rRNA signal in the corresponding sample. Percentage-change values for a given probe are based on the figures provided, and all experiments were repeated a minimum of two times. Before reprobing, blots were stripped twice in 40 mM Tris–HCl, pH 7.6 containing 0.1× SSC and 1.0% SDS at 80 °C for 20 min.

Results and discussion

Effects of insect herbivory on gene expression

Foliar tissues of *M. sativa* and the closely related plant *M. truncatula* contain saponins as a defense against insect herbivores (Tava and Odoardi, 1997; Huhman and Sumner, 2002). In response to caterpillar herbivory, the biosynthesis of the saponins medicagenic acid bidesmoside and soyasaponin I, that have negative effects on caterpillar physiological processes, are induced (Adel *et al.*, 2001; Agrell *et al.*, 2003, 2004). Terpenoid volatiles are also synthesized and released from *M. truncatula* plants that are fed upon by caterpillars (Leitner *et al.*, 2005; Korth, unpublished data). Both of these induced secondary metabolites share common early biosynthetic steps in the MVA and MEP pathways (Figure 1) (Eichenreich *et al.*, 2001). We examined the gene expression of pivotal enzymes in the MVA and MEP pathways in plants subjected to different herbivore treatments to understand the regulation of these pathways at a transcriptional level. EST homologues were selected from the *M. truncatula* database based on sequence similarity with known genes (Table 1).

The enzyme 1-deoxyxylulose-5-phosphate synthase catalyzes the first step of terpenoid biosynthesis in the MEP pathway; the condensation of pyruvate with the aldehyde of D-glyceraldehyde-3-phosphate forming 1-deoxy-D-xylulose 5-phosphate (DXP). This branch-point intermediate may be directed toward the biosynthesis of the B vitamins, pyridoxol (B6) or thiamine (B1), or into terpenoid biosynthesis (Julliard and Douce, 1991). The genes that encode this enzyme are members of a small gene family, of which two genes have been characterized in *M. truncatula* and it is believe that there are three members in *Arabidopsis* (Lange and Ghassemian, 2003; Walter *et al.*, 2002). *Mtdxs1* is a constitutive gene in the aerial tissues, whereas *Mtdxs2* is upregulated in roots in response to mycorrhizal symbiosis (Walter *et al.*, 2002).

The second enzyme in this pathway, 1-deoxyxylulose-5-phosphate reductoisomerase (DXR,

Table 1. Comparison between *M. truncatula* tentative consensus sequences and homologous genes at the nucleotide and protein levels.

Gene	Enzyme	*Medicago truncatula* vs.	Nucleotide identity (%)	Amino acid similarity (%)
dxs1	D-1-deoxyxylulose 5-phosphate synthase	*Lycopersicon esculentum*	79.1	89.9
dxr1	D-1-deoxyxylulose 5-phosphate reductoisomerase	*Arabidopsis thaliana*	86.1	92.6
Hmg1	3-hydroxy-3-methylglutaryl CoA reductase	*Solanum tuberosum*	53.2	71.2
tps1	terpene synthase	*Gossypium hirsutum*	46.7	66.4
Pgk	phosphoglycerate kinase	*Arabidopsis thaliana*	87.8	95.2

encoded by *Mtdxr1*) catalyzes the first committed step in the pathway, the reduction and isomerization of DXP to MEP, which is then converted to isopentenyl diphosphate through several catalytic steps (Schwender *et al.*, 1999; Carretero-Paulet *et al.*, 2002). In the cytosolic MVA pathway, the effect of herbivory on *Mthmg1* gene expression was examined. HMGR is a key regulatory enzyme catalyzing the irreversible reduction of 3-hydroxy-3-methylglutaryl CoA to MVA and is subject to regulation at many levels including transcription (Chappell, 1995). A putative sesquiterpene synthase gene, designated *Mttps1*, was chosen to represent an enzyme in the later steps of terpenoid biosynthesis (Gomez *et al.*, 2005).

Amounts of transcripts encoding enzymes early in the terpenoid biosynthetic pathways were lower in insect-damaged plants. In undamaged *M. truncatula* leaves, *Mtdxs1* expression was constitutive (Figure 2), which agrees with previous studies in *Arabidopsis thaliana*, *Lycopersicon esculentum* and *M. truncatula*, in which developing tissues, particularly leaves, had abundant *dxs* message (Estévez *et al.*, 2000; Lois *et al.*, 2000; Walter *et al.*, 2002). Mechanical damage or low levels of insect herbivory (Figure 2A) had minimal effects on *Mtdxs1* mRNA levels. However, a slight decrease of transcript levels was observed in tissues damaged by 5th instar *S. exigua* and this was accentuated in plants exposed to more severe insect infestation (Figure 2B). There is the possibility that another member of the small gene family, *Mtdxs2*, is up-regulated in response to biotic interactions such as caterpillar herbivory, as is the observed in the root during *Medicago*-mycorrhizal associations (Walter *et al.*, 2002).

In comparison with *Mtdxs1*, *Mtdxr1* transcript levels were greatly reduced in response to insect herbivory. Transcript levels in tissues damaged by herbivory, either by 3rd or 5th instar larvae, had

reductions of 34 or 52%, respectively, of the transcript amounts in undamaged leaves (Figure 2). A decrease (around 25%), albeit not so pronounced, was also observed in systemic tissues, suggesting modulation of gene expression in distal tissues. However, transcript accumulation was not affected in mechanically wounded leaves, damaged
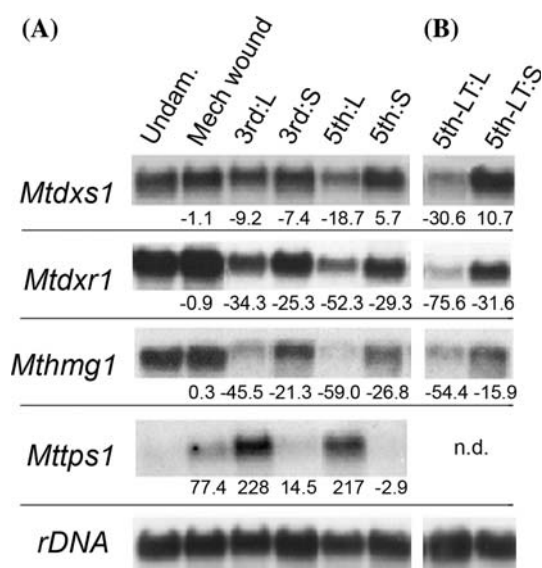

Figure 2. Medicago trunctula gene expression in response to mechanical damage and insect herbivory by different *S. exigua* stages and infestation levels. RNA was isolated from damaged (L) and non-damaged (systemic (S)) leaves from mechanically damaged (Mech. wound) and insect-damaged leaves. *S. exigua* larvae (either 3rd or 5th instars) were placed on plants for (A) 1 h in the short-term experiments or (b) 6 h in the long-term (LT) experiments. Blots were hybridized with probes for genes in the MEP pathway (*Mtdxs1, Mtdxr1*), the MVA pathway (*Mthmg*), terpene synthase (*Mttps1*) and 25S ribosomal DNA (*rDNA*). Measurements were repeated at least three times for each treatment and a typical result is shown. Numbers below each band represent the percentage difference in signal intensity compared to the signal in the undamaged sample for a given probe.

either by cutting with scissors or with a tracing wheel, and remained at constitutive levels found in undamaged leaves (Figures 2 and 5). In artificially damaged leaves, transcript levels of *Mthmg1* were not affected by mechanical damage and remained at constitutive levels (Figures 2 and 5). However, *Mthmg1* transcript levels were much lower in leaves damaged by insect herbivory compared with control plants or systemic leaves of insect-infested plants (Figure 2). In comparison to the genes encoding enzymes in early steps of the terpenoid biosynthetic pathways, *Mttps1* transcript levels were strongly induced in plant tissues damaged by mechanical wounding or caterpillar herbivory (Figure 2). Transcript levels of *Mttps1* are also upregulated in response to treatment of *M. truncatula* plants with MeJA or caterpillar regurgitant (Gomez *et al.*, 2005). As a control, gene transcript levels of phosphoglycerate kinase, an enzyme involved in primary metabolism, were also monitored; constitutive expression was not affected by wounding or insect herbivory (data not shown).

In response to caterpillar herbivory, *de novo* terpenoid volatile biosynthesis and release occurs on a shorter time frame (within hours) than saponin biosynthesis (1 week after herbivory) (Agrell *et al.*, 2003, 2004; Leitner *et al.*, 2005). Therefore, the changes in terpenoid gene expression observed in these experiments may be related to the biosynthesis of terpenoid volatiles, rather than saponins. However, transcripts encoding key enzymes in *M. truncatula* saponin biosynthesis have been shown to be induced in cell cultures within 12 h of methyl jasmonate treatment (Suzuki *et al.*, 2002).

Developmental stage of Lepidopteran larvae has been shown to influence plant volatile profiles (Takabayashi *et al.*, 1995). The distinct feeding behaviour of different larval instars may influence the plant's defense responses. Younger *S. exigua* instars (1st and 2nd instars) scrape *M. truncatula* plant tissue between the leaf veins leaving a scarified appearance, 3rd instar larvae tend to eat tissue between the veins producing a boxy pattern compared with later instars (4th–6th) that eat across the leaf in a circular pattern. Parasitic females wasps, *Cotesia kariyai*, were more attracted to semiochemicals released from maize seedlings damaged by younger instars (1st to 4th) than older instars of the true armyworm, *Pseudoletia unipunctata* and the factor(s) responsible

for altering plant volatiles was traced to the insect's oral secretions (Takabayashi *et al.*, 1995). Parasitism of these early larval stages is beneficial to the parasitoid, as it allows sufficient time for development and to the plant as herbivory by parasitized caterpillars is greatly reduced (Takabayashi *et al.*, 1995, Turlings and Fritzsche, 1999). At the transcript level, differential plant responses to insect instar were not observed in these experiments, despite different feeding patterns of younger vs. older larvae.

Insect herbivory often elicits different plant responses than artificial wounding of leaves (Walker-Simmons *et al.*, 1984; Korth and Dixon, 1997; Reymond *et al.*, 2000). In potato leaves, wound-inducible transcripts accumulate more rapidly in response to herbivore damage than mechanical wounding (Korth and Dixon, 1997). The lack of change in *Mtdxr1* or *Mthmg1* gene expression in response to mechanical damage, as compared with lower transcript levels observed in response to insect herbivory, may be due to differences in the type of damage, even though we attempted to mimic herbivory with mechanical damage. Another possible cause for the observed differences following biotic or abiotic damage is the absence of an insect-derived factor(s) contacting the mechanically damaged leaves. Continuous mechanical robotic damage over a longer time period has been used to show that release volatile signals are similar to those from insect damaged plants, however, the ratios of individual terpenes released are very different among mechanically and insect damaged plants (Mithöfer *et al.*, 2005).

Role of salivary gland factors

To determine the role of salivary factors in mediating plant responses, the effects of herbivory by caterpillars with normal or reduced levels of labial gland secretions were compared. Cauterization of the spinneret, a specialized structure from which labial saliva is released, impairs GOX secretion as confirmed by the lower levels of H_2O_2 produced by cauterized insects tested via the DAB assay (Figure 3). Herbivory by insects with cauterized spinnerets caused small increases in transcript levels of *Mtdxs1* and *Mthmg1*, whereas cauterization did not affect induction of *Mttps1*, when compared to herbivory by normal (Mock) insects (Figure 4). Therefore, changes in
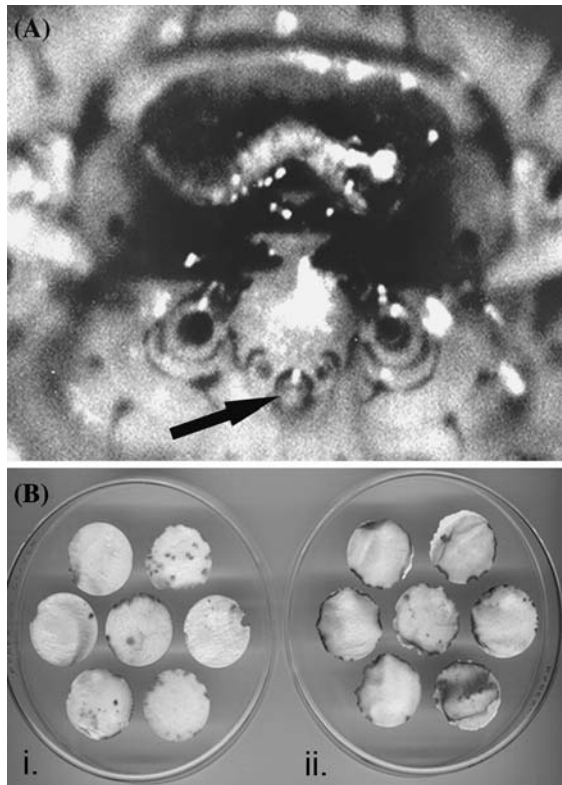
Figure 3. (A) 5th instar *Spodoptera exigua* larva with an arrow identifying the spinneret. (B) Glass fiber disks damaged by *S. exigua* caterpillars and stained with horseradish peroxidase (HrP) and 3,3′-diaminobenzidine tetrahydrochloride (DAB). The presence of dark brown precipitate indicates that the insects are able to secrete salivary GOX and the cauterization was ineffective (ii. Mock) compared to insects which have undergone a successful cauterization (i. Caut).

Figure 4. Medicago truncatula gene expression in response to herbivory by *Spodoptera exigua* larvae with cauterized spinnerets. RNA was purified from systemic (S) and damaged (L) leaf tissue of 6-week-old plants that were infested with insects that had cauterized spinnerets (Caut.) or with "mock-treated" insects (Mock). Blots were hybridized with probes for genes in the MEP pathway (*Mtdxs1, Mtdxr1*), the MVA pathway (*Mthmg*) and terpene synthase (*Mttps1*) and 25S ribosomal DNA (*rDNA*). Measurements were repeated at least three times for each probe and a typical result is shown. Numbers below each band represent the percentage difference in signal intensity compared to the signal in the corresponding "mock-treated" sample for a given probe.

accumulation of transcripts for these genes in response to insect herbivory may be either a wound-specific response or due largely to other insect elicitors not blocked by spinneret cauterization, such as volicitin found in regurgitant.

Transcript levels of *Mtdxr*1 in local leaves were consistently lower in plants damaged by normal insects than in plants chewed upon by insects with cauterized spinnerets (Figure 4). This indicates that a labial gland salivary factor is largely responsible for the decrease in transcript levels occurring in response to insect herbivory. However, levels of *Mtdxr*1 transcripts in locally wounded leaves damaged by insects with cauterized spinnerets were slightly lower than undamaged or systemic leaves (−17.3%), indicating that a decrease of transcript accumulation still occurs in response to these
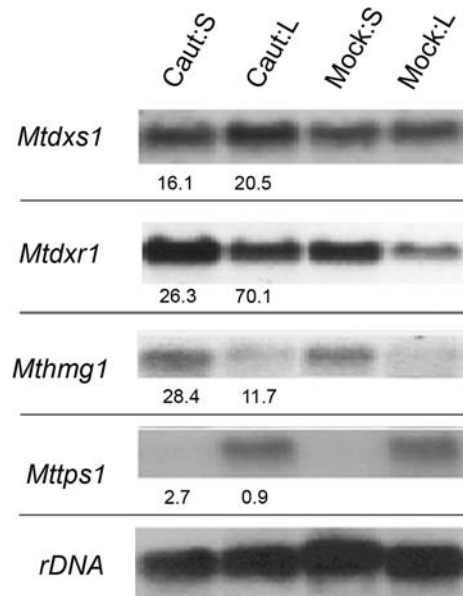
insects. Therefore, other insect-derived elicitors in the mandibular saliva or regurgitant may also be involved in suppression of transcript levels following *S. exigua* herbivory. Schittko *et al.* (2001) detected reduction in transcript levels of five genes in *Nicotiana attenuata* leaves treated with crude tobacco hornworm regurgitant, although none of the genes examined were predicted to encode enzymes in the terpenoid pathways. The complexity of plant responses to insect factors is illustrated by the observation that application of regurgitant resulted in the suppression of gene transcripts that were either up- or down-regulated in response to mechanical wounding alone (Schittko *et al.*, 2001). This is in contrast to previous observations that levels of other wound-induced gene transcripts are enhanced by addition of regurgitant (Korth and Dixon, 1997; Frey *et al.*, 2000; Schittko *et al.*, 2001; Gomez *et al.*, 2005).

Role of glucose oxidase (GOX) and hydrogen peroxide (H₂O₂)

Salivary GOX has been implicated in the suppression of induced plant defenses; the H_2O_2 produced by salivary GOX might interfere with plant defenses through modulation of signaling responses such as the JA or ethylene pathways (Musser *et al.*, 2002). To address the nature of the salivary factor involved in suppressing transcript levels, GOX or its enzymatic product, H_2O_2, were applied to wounded *M. truncatula* leaves and transcript levels were measured (Figure 5). Treatment of *M. truncatula* leaves with GOX or H_2O_2 had minimal effect on *Mtdxs1, Mthmg1* and *Mttps1* transcript levels. In comparison, application of GOX or H_2O_2 to mechanically damaged leaf tissue resulted in lower *Mtdxr1* transcript levels than with wounding alone, suggesting that H_2O_2 produced by salivary GOX is the active factor eliciting this response. The effects of addi-

tion of H_2O_2 to leaves confirmed that the reduction in transcript levels is attributed to GOX activity rather than to contaminating enzymes present in the commercial GOX preparation. Transcripts for *Mtdxr1* are abundant in undamaged leaves and mechanical damage or herbivory by insects with cauterized spinnerets do not reduce transcript levels (Figure 4). Therefore, in this case, the decrease in transcript levels after addition of GOX is most likely due to direct effects of H_2O_2 and not from interference with wound-inducible, JA-dependent gene expression. Although H_2O_2-generating GOX contributes to regulation of defensive transcripts, there are likely other oral factors that also play a role as signals to the plant of biotic damage. Measurement of defense-related transcripts in *Nicotiana attenuata* showed that although GOX and/or H_2O_2 can affect gene expression, they play a relatively minor in eliciting defense responses in that system, as compared to the effects of other insect oral factors such as fatty acid-amino acid conjugates (Halitschke *et al.*, 2003). Our observations that *Mthmg1* and *Mtdxs1* transcript levels are suppressed by insect feeding, but not by GOX or H_2O_2, again suggest that there are other factors involved in the specific negative regulation of plant defense genes. In particular, suppression of *Mthmg1* is likely occurring in response to wound events associated with feeding and/or elicitors originating somewhere other than in the labial salivary glands because transcripts are still strongly suppressed even after damage by insects with cauterized spinnerets (Figure 4). On the contrary, transcripts for *Mtdxs1* are suppressed by insects with impaired spinnerets but not by GOX or H_2O_2 treatments (Figure 5), suggesting that other factors in labial gland saliva can also play a role in gene suppression.
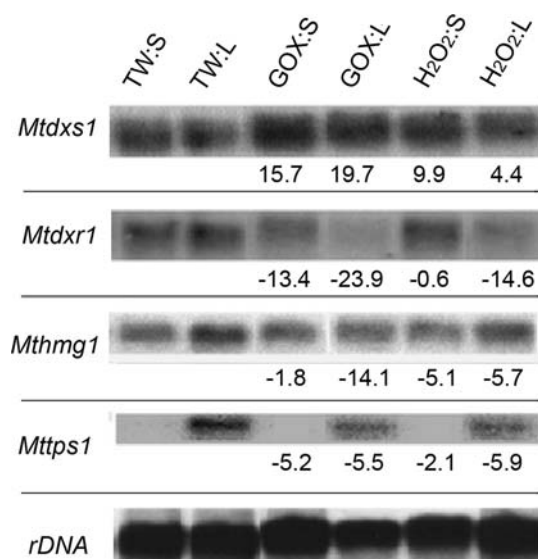

Figure 5. Medicago truncatula gene expression in leaves treated with glucose oxidase (GOX) or hydrogen peroxide (H₂O₂). RNA (15 μg) was purified from systemic (S) and damaged (L) leaves from plants wounded with a tracing wheel and treated with sodium phosphate buffer, pH 7.0 (TW), GOX (0.0015 U) or H_2O_2 *(22.5 nM)*. Blots were hybridized with probes for genes in the MEP pathway (*Mtdxs1, Mtdxr1*), the MVA pathway (*Mthmg*) and terpene synthase (*Mttps1*) and 25S ribosomal DNA (*rDNA*). Measurements were repeated at least twice for each probe and a typical result is shown. Numbers below each band represent the percentage difference in signal intensity compared to the signal in the corresponding TW sample for a given probe.

Conclusion

The strict regulation of terpenoid production in plants may reflect metabolic costs associated with biosynthesis of secondary metabolites (Gershenzon, 1994). Our data clearly support the idea the DXR is a critical enzyme in the MEP pathway and levels of gene expression are tightly constrained by insect herbivory. In ripening tomato fruit, the correlation between *dxs* gene expression and carotenoid production suggests that DXS is a

key regulatory enzyme (Lois *et al.*, 2000; Rodrí-guez-Concepción *et al.*, 2001). In comparison, transgenic peppermint with overexpressed or cosuppressed DXR showed dramatically altered levels of monoterpene essential oil composition, establishing the pivotal role of DXR in the regulation of terpenoid biosynthesis (Mahmoud and Croteau, 2001). In *M. truncatula*, *Mtdxs1* and *Mtdxr1* are constitutively expressed in leaf tissue. In response to insect herbivory, transcript levels of *Mtdxs* were slightly reduced in infested plants whereas a dramatic decrease in *Mtdxr* message levels were observed, suggesting that transcript regulation of *Mtdxr1* is a mechanism for regulating terpenoid flux through the MEP pathway (Figure 2).

Suppression of genes predicted to encode enzymes in the terpenoid pathways following herbivory seems somewhat contradictory, as the *de novo* biosynthesis and release of volatile terpenes seems to be a ubiquitous response of insect-damaged plants (Paré and Tumlinson, 1999; Dicke and van Loop, 2000; Kessler and Baldwin, 2002), including *M. truncatula* (Leitner *et al.*, 2005; K. Korth, unpublished data). Although the quantities of transcripts encoding early enzymes in both terpenoid pathways are reduced following herbivory, those encoding later steps (e.g. *Mttps*1) clearly increase following biotic or abiotic damage. Whereas some genes and enzymes in the terpenoid pathways are induced following herbivory (Korth and Dixon, 1997; Bouwmeester *et al.*, 1999; Van Poecke *et al.*, 2001; Gomez *et al.*, 2005), other studies have confirmed our observations of transcript suppression by insect damage. In *Arabidopsis*, wounding suppresses transcripts encoding HMGR and farnesyl diphosphate synthase (Reymond *et al.*, 2000). Transcript levels of a significant numbers of *Arabidopsis* genes are reduced in response to salicylic acid, methyl jasmonate, or ethylene treatment (Schenk *et al.*, 2000). For genes encoding early enzymes in both pathways (*i.e.*, *Mtdxr1* and *Mthmg1*), reduced transcript levels were only observed in local tissues; this infers that herbivory does not interfere with IPP biosynthesis in systemic leaf tissue. Our data are reminiscent of results observed in insect-damaged poplar, where differential regulation of early and late steps in each of the terpenoid pathways was observed (Arimura *et al.*, 2004a). Transcripts encoding terpene synthases and farnesyl diphosphate synthase in poplar trees are induced by forest tent caterpillar (*Malacosoma disstria*) feeding; however, in that study, levels of transcripts encoding DXR and HMGR did not change. These combined findings suggest an important role for transcriptional regulation of terpene synthases in regulating the specificity of volatile terpene emissions. The induction of terpene synthases in response to insect feeding has been previously suggested to be indicative of an active defense. A good example of this has been reported in Sitka spruce (*Picea sitchensis*), where induction of multiple terpene synthase genes is accompanied by the synthesis and release of volatile defensive terpenes (Miller *et al.*, 2005). In addition, several studies have indicated a concomitant increase in terpene synthase transcripts and the release of terpene volatiles in response to herbivory by mites, in cucumber (Mercke *et al.*, 2004) and *Lotus japonicus* (Arimura *et al.*, 2004b). In *M. truncatula*, at least four genes encoding terpene synthases are strongly induced by lepidopteran herbivory (Gomez *et al.*, 2005). Currently, we are examining the possibility that key regulatory genes in the MVA and MEP pathways are members of multigene families subject to differential spatial and temporal expression.

It is apparent that insect-derived oral elicitors, in addition to wounding, contribute to the complex signaling between plant and insect. Our data indicate that this complexity is also reflected in the differential regulation of early and late steps within the same biosynthetic pathways leading to production of defensive compounds.

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