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Lepidopteran Herbivory and Oral Factors Induce Transcripts Encoding Novel Terpene Synthases in *Medicago truncatula*

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Terpenes are an important class of defense compounds that accumulate in plants after pathogen infection or arthropod injury. Sequences predicted to encode terpene synthases were selected from an expressed sequence tag (EST) database of *Medicago truncatula*. Four putative terpene synthase clones (*MtTps*1– *MtTps*4), originating from a chewing insect-damaged *M. truncatula* leaf cDNA library, were isolated. Transcript levels of each gene examined increased in response to artificial wounding, *Spodoptera exigua* herbivory, and treatment with volatile methyl jasmonate (meJA). Addition of *S. exigua* regurgitant to wound sites triggered transcript accumulation of *MtTps*1 and levels increased with higher concentrations of regurgitant. Furthermore, induction of *MtTps*1 occurred after application of *N*-linolenoyl-glutamate or *N*-linoleoyl-glutamate, factors found in lepidopteran regurgitant. Genomic DNA blots indicate that each of the putative proteins is encoded by a single-copy gene or a small gene family. Proteins encoded by *MtTps*3 and *MtTps*4 are imported into the soluble fraction of chloroplasts in in vitro assays, whereas proteins encoded by *MtTps*1 and *MtTps*2 are not imported into chloroplasts. Combined with sequence comparisons of multiple plant terpene synthases, the import data indicate that *MtTps*1 and *MtTps*2 likely encode sesquiterpene synthases and that *MtTps*3 and *MtTps*4 encode mono- or di-terpene synthases. In addition to serving as a valuable model legume species for genomic studies, *M. truncatula* should prove a valuable source of novel terpene-producing enzymes. Induction of wound-responsive genes by insect oral factors suggests that *M. truncatula* senses biotic damage through the presence of elicitors originating in the herbivore. Arch. Insect Biochem. Physiol. 58:114–127, 2005. © 2005 Wiley-Liss, Inc.

Keywords: chloroplast; isoprenoid; oral secretion; terpenoid; wound

INTRODUCTION

Plants exhibit a number of physiological and biochemical responses to physical stimuli such as wounding, and chemical stimuli such as elicitor compounds from pathogens or herbivores. Among these responses is the systemic synthesis and release of volatile organic compounds from leaves following injury by arthropod herbivores (reviewed by Dudareva et al., 2004). These volatile com-

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Abbreviations used: EST, expressed sequence tag; FAC, fatty-acid:amino-acid conjugate; 18:2-GLN, *N*-linoleoyl-glutamine; 18:3-GLN, *N*-linolenoyl-glutamine; 18:2-GLU, *N*-linoleoyl-glutamate; 18:3-GLU, *N*-linolenoyl-glutamate; GOX, glucose oxidase; IPP, isopentenyl diphosphate; JA, jasmonic acid; meJA, methyl jasmonate; MEP, 2C-methyl erythritol 4-phosphate; *MtTps, Medicago truncatula* terpene synthase; MVA, mevalonate.

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pounds can attract natural enemies of the herbivores (Dicke and Sabelis, 1988; Turlings et al., 1990), thereby indirectly protecting the plant from damage through an induced mechanism (Kessler and Baldwin, 2001). The induction of transcripts encoded by specific suites of genes is observed following insect herbivory or treatment with insect-derived oral factors, suggesting that induced defenses can be regulated at the level of transcription (reviewed by Korth, 2003).

Terpenes are one of the most abundant and common classes of induced volatile plant compounds released in response to insect herbivory. The structures of terpenes are based on a five-carbon building block, isopentenyl diphosphate (IPP). Two independent biosynthetic pathways can produce IPP, the mevalonate (MVA) pathway localized in the cytosol and the 2*C*-methyl erythritol 4-phosphate (MEP) pathway found in plastids (Rohmer, 1999). The structure of the terpene that is ultimately produced by the plant is determined by the activities of specific terpene synthase enzymes. Mono- and di-terpenes (C_{10} and C_{20} compounds, respectively) are thought to originate in plastids via the MEP pathway, whereas sesqui-, tri- (C_{15} and C_{30}, respectively), and poly-terpenes are produced predominantly by the MVA pathway (reviewed by Lichtenthaler, 1999). For the induced de novo production of volatile compounds (ParÉ and Tumlinson, 1997), expression of specific genes involved in terpenoid biosynthesis, along with terpene synthase activities, have been shown to increase (e.g., Bouwmeester, et al., 1999; Schnee et al., 2002; Arimura, et al., 2004).

Terpenoids have a wide range of activities and their applications include flavorings, fragrances, food additives, cosmetics, and pharmaceuticals (Mahmoud and Croteau, 2002). Individual sesquiterpenes have been shown to act as antimicrobial phytoalexins and as insect antifeedants. Triterpenes such as the saponins can have antifeedant and antifungal properties that aid in plant defense (Haralampidis et al., 2001). Terpenes derived from either the cytosolic MVA pathway or the plastid MEP pathway can play roles in plant-pest interactions, often functioning in plant defense (Chappell, 1995).

Differential responses in plants to mechanical damage and insect damage are at least partially due to perception of insect-derived oral factors during herbivory. There are several examples of elicitors originating in insect oral secretions. Regurgitant from caterpillars of several species as well as from the grasshopper *Schistocerca americana* induce the release of significant amounts of volatile organic compounds from corn seedlings (Turlings et al., 1993). Herbivore-specific elicitors have been isolated from the oviposition fluid of weevils (Doss et al., 2000) but their introduction into plants is not directly associated with feeding events. A protein component in the saliva of some insects, glucose oxidase (GOX), can trigger responses by the plant (Felton and Eichenseer, 1999) and suppress plant defenses (Musser et al., 2002). "Volicitin" (*N*-17-hydroxylinolenoyl-L-glutamine), an elicitor isolated from *Spodoptera exigua* oral secretions, induces similar plant responses as *S. exigua* herbivory (Alborn et al., 1997). Volicitin is a fatty-acid: amino-acid conjugate (FAC), where the fatty acid moiety is derived from the host plant and the conjugation of glutamine occurs in the caterpillar gut (Paré et al., 1998; Lait et al., 2003). Volicitin-treated plants become more attractive than untreated plants to natural enemies of the herbivorous insects (Alborn et al., 1997; Turlings et al., 2000). Volatile-inducing non-hydroxylated linolenic and linoleic acids conjugated with either glutamine or glutamate are also abundant in the regurgitant of tobacco hornworm, *Manduca sexta*, and other lepidopteran species (Pohnert et al., 1999; Halitschke et al., 2001; Alborn et al., 2003).

In addition to the responses to insect-derived factors, plant defenses can be triggered by stress-induced increases in methyl jasmonate (meJA), a volatile form of the plant hormone jasmonic acid (JA) (Creelman and Mullet, 1997). Lima bean (*Phaseolus lunatus*) treated with JA, meJA, and spider mites (*Tetranychus urticae*) emit more volatiles than uninfested plants, resulting in attraction of predatory mites (*Phytoseiulus urticae*) (Dicke et al., 1999). The volatile-inducing activity of JA can also be dependent on interactions with insect oral factors and/or other plant hormones such as ethylene (Schmelz et al., 2003).

A growing set of genetic and genomic tools have made *Medicago truncatula* an increasingly important and useful model system (Cook, 1999). This self-fertilizing legume possesses a relatively small diploid genome and has a short generation time. In addition, a large-scale genomics project in *M. truncatula* is underway, including the sequencing of expressed mRNAs. To date, nearly 190,000 expressed sequence tags (ESTs) are available in a public database (http://www.tigr.org/tdb/mtgi/); including almost 10,000 ESTs that were derived from *S. exigua*-injured *M. truncatula*.

Based on sequence similarities with known genes from other plant species, we selected four putative terpene synthase cDNA clones as potential targets for future efforts on understanding insect-induced terpene biosynthesis in *Medicago*, from the *M. truncatula* EST database. We characterized protein localization and transcript accumulation patterns of these putative terpene synthases in response to physical damage and biotic signals.

MATERIALS AND METHODS

Plant and Insect Maintenance

M. truncatula cv. Jemalong, line A17, was maintained under standard conditions in a growth chamber at 24°C with a 16:8-h light:dark regimen. Fertilizer was administered at 2-week intervals. All treatments were carried out in a greenhouse and were started at 0900 hours. Eggs of *S. exigua* were obtained from the USDA Gast Rearing Lab (Starkville, MS). Larvae were maintained on an artificial diet at approximately 22°C.

DNA clones

Plant cDNA clones were derived from a library produced from fully expanded *M. truncatula* leaves of plants fed upon by *S. exigua* (beet armyworm) for 24 h. For library production, insect-wounded local and systemic (undamaged leaves from injured plants) leaves were harvested and pooled. The library was subjected to EST sequencing at the Samuel Roberts Noble Foundation (Ardmore, OK). Automated annotation was based on sequence similarity with characterized terpene synthases from other plant species and ESTs were identified in a search for terpene synthase clones in the *M. truncatula* EST database (http://www.tigr.org/tdb/tgi/mtgi). The terpene synthase designations and the Genbank accession numbers for the EST sequences are: *MtTps*1 (accession no. BF640252); *MtTps*2 (accession no. BI265673), *MtTps*3 (accession no. BF639687); and *MtTps*4 (accession no. BG449363). Four individual clones were chosen for analysis, obtained from the repository at the Noble Foundation (Ardmore, OK), and the full-length sequence of each cDNA clone was determined using standard methods. The Genbank accession numbers for the resulting full-length cDNA sequences are: *MtTps*1 (accession no. AY763425), *MtTps*2 (accession no. AY766250), *MtTps*3 (accession no. AY766249), and *MtTps*4 (accession no. AY766248).

Sequence Analysis

The four clones from *M. truncatula* were compared to 19 known terpene synthase sequences derived from angiosperms and available in Genbank. Species names, terpene product, and accession number for each are: *Artemisia annua*, amorpha-4,11-diene synthase (CAB94691), *A. annua*, 8-epicedrol synthase (AAF80333), *Lycopersicon esculentum*, sesquiterpene synthase (AAG41889), *L. hirsutum*, sesquiterpene synthase (AAG41891), *Cichorium intybus*, germacrene A synthase (AAM21658), *Capsicum annuum*, 5-epi-aristolochene synthase (CAA06614), *Nicotiana tabacum* , 5-epi-aristolochene synthase (AAA19216), *Solanum tuberosum*, vetispiradiene synthase (BAA82141), *L. esculentum*, vetispiradiene synthase (AAG09949), *Gossypium hirsutum*, delta-cadinene synthase (AAC12784), *Salvia officinalis*, 1,8-cineole synthase (AAC26016), *Mentha longifolia*, limonene synthase (AAD50304), *S. officinalis*, sabinene synthase (AAC26018), *S. officinalis*, bornyl diphosphate synthase (AAC26017), *A. annua*, beta-pinene synthase (AAK58723), *Arabi-*

dopsis thaliana, ent-kaurene synthase (AAC39443), *Cucurbita maxima*, ent-kaurene synthase B (AAB39482), *Stevia rebaudiana*, kaurene synthase (AAD34295), and *Pisum sativum*, ent-kaurene synthase (AAB58822). Amino acid sequences were aligned with ClustalW. Sequence distances were calculated using "Protdist" and an unrooted tree was constructed via the UPGMA method in the "Neighbor" program; both programs are contained in PHYLIP version 3.61 (http://evolution.genetics.washington.edu/phylip.html). The selected genes represent sequences from seven different dicot families.

TargetP analysis (http://www.cbs.dtu.dk/services/TargetP/) was used to test sequences for the presence of predicted chloroplast transit peptide regions. Amino acid sequence identity and similarity percentages among the *M. truncatula* terpene synthases, not considering the N-terminus transit peptide signals, were compared via the Gap program within the GCG SeqWeb software.

Plant Treatments

Third-instar *S. exigua* larvae were placed on intact plants and allowed to roam on the plant placed within a cage. Larvae were allowed to feed for 6 h before leaf samples were collected. Leaves that had been damaged by the insect were "local," and undamaged leaves nearby an insect-damaged leaf were "systemic." Artificial wounding was administered with a tracing wheel (1.5-cm diameter) that created a linear series of puncture wounds.

For meJA treatments, intact plants were placed in 18-L glass chambers. Cotton swabs with 1.0 µL meJA in 50 µL ethanol (calculated volatile concentration 0.25 µM) were placed in each chamber and the open-end bottom of each chamber was covered with a layer of foil and cheesecloth. Negative-control plants were treated the same, with solvent alone added to the cotton swab. The plants were removed from the chambers after 1 h and leaf samples were taken at the indicated times over an 18-h time period.

Nucleic Acid Analysis

Leaves were collected, immediately chilled in liquid nitrogen, and stored at –75°C until analysis. Total RNA was extracted using TriReagent (MRC, Inc. Cincinnati, OH) and separated on 1% agarose formaldehyde gels. The RNA was transferred to nylon membranes and hybridized with labeled probes (Church and Gilbert, 1984). Insert DNA from individual cDNA clones was amplified via polymerase chain reaction and radio-labeled with ^{32}P in random-primer reactions (Sambrook et al., 1989).

M. truncatula genomic DNA was isolated according to Junghans and Mettzlaff (1990). DNA was digested in individual reactions with *Bam*HI or *Eco*RI overnight at 37°C. Cleaved genomic DNA was separated on a 0.8% agarose gel, denatured, and transferred to a nylon membrane (Sambrook et al., 1989). Hybridizations were carried out as for RNA blots.

Protein Import Assay Using Isolated Chloropalsts

Chloroplast protein import assay was conducted as described (Inoue and Keegstra, 2003). Briefly, radio-labeled precursor proteins were prepared from the individual terpene synthase clones with [^{35}S]methionine (Amersham Biotech Pharmacia, Piscataway, NJ) and T$_N$T® Coupled Reticulocyte Lysate Systems (Promega, Madison, WI). An aliquot of each translation product was incubated with chloroplasts isolated from pea seedlings containing 12.5 µg chlorophyll and 3 mM Mg-ATP in the import buffer (50 mM Hepes-KOH, 330 mM sorbitol, pH 8.0) at room temperature under light for 20 min. Intact chloroplasts were reisolated, washed, and directly analyzed, or incubated in the import buffer with or without 1.25 µg trypsin (Sigma, St. Louis, MO) on ice for 30 min under dark conditions. The proteolysis reaction was quenched with 1.25 µg of trypsin inhibitor (Sigma), then chloroplasts were re-purified, washed, lysed hypotonically, and centrifuged at 100,000*g* at 4°C for 10 min to obtain the supernatant and the membrane fractions. Each fraction was resuspended in sample buffer, proteins were separated by SDS-PAGE, and ^{35}S-labeled bands were visualized using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Under these conditions, a chloroplast stromal protein, the small subunit of ribulose bisphosphate carboxylase/oxygenase, was recovered in the supernatant fraction and resistant to the protease, whereas Tic22, an inner envelope protein facing the intermembrane space (Kouranov et al., 1998), was recovered in the membrane fraction and susceptible to the protease (data not shown).

Crude Regurgitant of *Spodoptera exigua*

Regurgitant from *M. truncatula*-fed *S. exigua* was collected, on ice, by mild aspiration from 3rd to 4th instar larvae as described (Alborn et al., 1997). Tubes with pooled regurgitant were placed immediately in a boiling water bath for 5 min to destroy elicitor-degrading activities (Mori et al., 2001), centrifuged to remove particulates, and stored at –20°C. Regurgitant was diluted 1:10, 1:50, and 1:500 in 5 mM sodium phosphate buffer, pH 8.0, and applied to five fully expanded *M. truncatula* trifoliates per plant. Tween 20 (ICN Biomedicals Inc., Aurora, OH) was added to each tube at a concentration of 0.067% as a surfactant. Two leaves of each trifoliate were wounded using a tracing wheel and 25 µl of each solution was split and applied to wound sites. Sodium phosphate buffer containing Tween 20 was applied as control treatment. The experimental plants were 78 days old when the experiment was conducted. Plants remained in the greenhouse for 6 h (1030 to 1630) before the treated trifoliates were collected and placed in liquid nitrogen.

Lepidopteran FAC Elicitors

Synthetic elicitors, selected so that all combinations of 18:2/18:3 fatty acids and GLN/GLU could be tested, were prepared as described (Alborn et al., 2003). Elicitors *N*-linoleoyl-glutamine (18:2-GLN), *N*-linolenoyl-glutamine (18:3-GLN), *N*-linoleoyl-glutamate (18:2-GLU), and *N*-linolenoyl-glutamate (18:3-GLU) were applied to wound sites on five *M. truncatula* trifoliates per plant. The elicitors were dissolved in 5 mM phosphate buffer, pH 8.0, at a concentration of 80 ng/µL. *M. truncatula* plants were 25 days old and treated as for regurgitant applications. Plants remained in the greenhouse for 6 h (1030 to 1630) before the treated leaves were collected and placed in liquid nitrogen. Transcript levels of *MtTps*1 were analyzed as indicated previously.

RESULTS

EST Clone Selection

A search of the nearly 190,000 clones in the *M. truncatula* EST database revealed the presence of at least four putative terpene synthase clones that were derived from insect-damaged leaves. Annotation of the clones is automated and putative gene functions are assigned and available in the searchable database (http://www.tigr.org/tdb/tgi/mtgi). Assignment of the clones was based on the presence of highly conserved sequence domains for terpene synthases. The selected clones were designated *MtTps*1–4. Clones *MtTps*1, *MtTps*2, and *MtTps*4 were found only among sequences from the herbivore-damaged leaf library, and not in sequences derived from any of the other multiple tissues or treatments represented in the extensive *M. truncatula* EST database. Clones for *MtTps*3 were also found in libraries derived from fungal-infected leaves, nodulated roots, and developing seed pods. Full-length cDNA clones for each sequence were obtained. The predicted length of peptide sequences of *Mt*TPS1-4 were 562, 553, 573, and 580 amino acids, respectively.

Sequence Analysis

Analysis with the ChloroP program (Emanuelsson et al., 1999) indicates that the *MtTps*3 and *MtTps*4 sequences are each predicted to encode a plastid transit signal at the amino terminus. Neither the *MtTps*1 nor the *MtTps*2 sequences contain a predicted plastid transit sequence. The predicted amino acid sequence of *Mt*Tps1 and *Mt*Tps2 showed the highest similarity to known sesquiter-

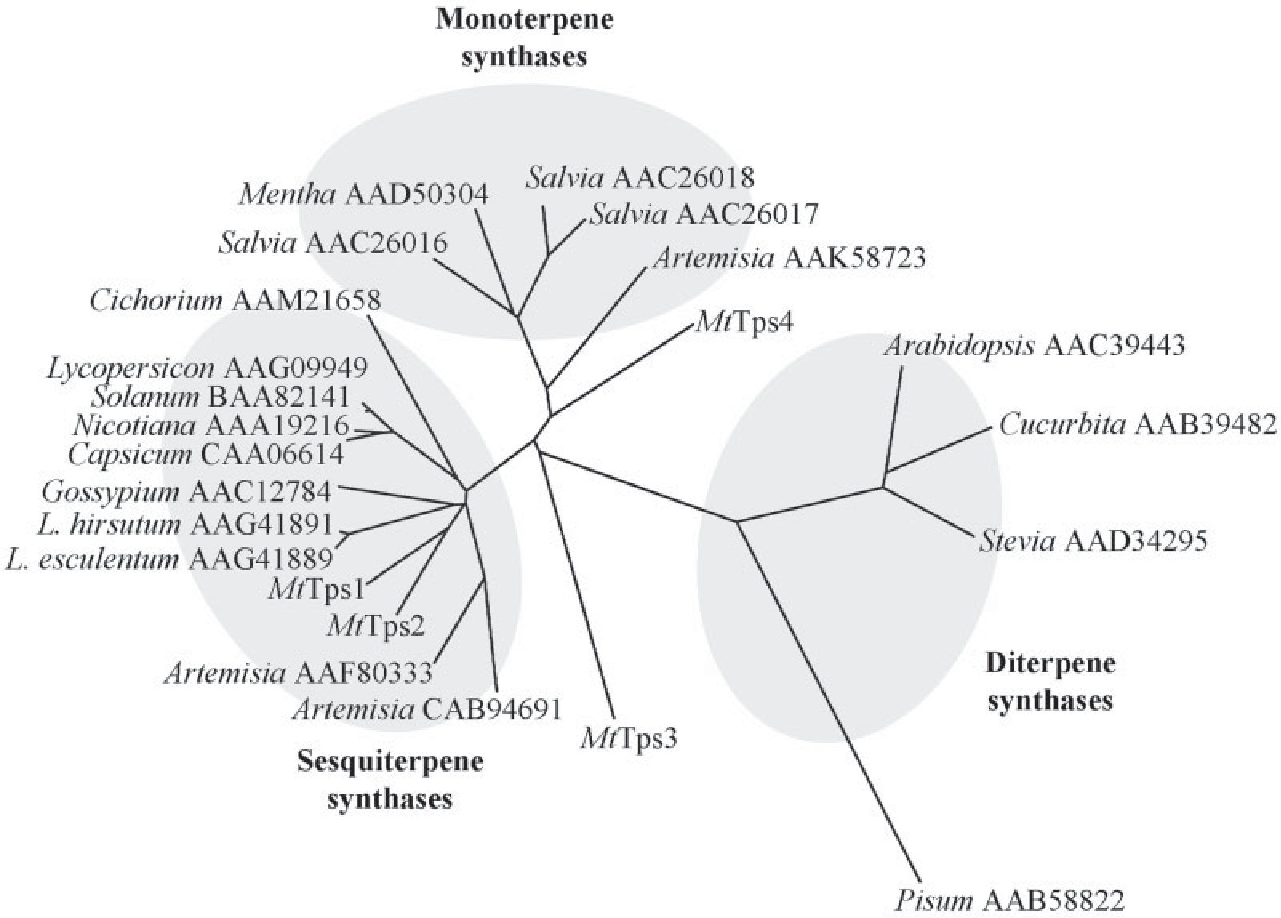

Fig. 1. Amino acid sequence similarities of deduced full-length sequences of angiosperm terpene synthases. Sequences were aligned using ClustalW and protein distances calculated with Protdist via nearest neighbor-joining method. *Mt*TPS1 and *Mt*TPS2 bear the highest sequence similarity to known sesquiterpene synthases. *Mt*TPS4 and *Mt*TPS3 cluster most closely with known mono- and diterpene synthases, respectively.

pene synthases from angiosperms of the genera *Artemisia*, *Capsicum*, *Cichorium*, *Gossypium*, *Lycopersicon*, *Nicotiana*, and *Solanum* (Fig. 1). *Mt*Tps4 has highest sequence similarity to known monoterpene synthases of the genera *Artemisia*, *Salvia*, and *Mentha* (Fig. 1). The predicted amino acid sequence of *Mt*Tps3 does not group closely with any of the characterized mono- or di-terpene synthases. A number of known terpene synthases from conifer species were also included in initial sequence comparison analyses, but these consistently grouped as a distinct cluster (data not shown) and were omitted in the final analysis. It is noteworthy that the genes tend to cluster according to enzymatic function, rather than by plant family.

Comparison of the sequence identity and similarity among *Mt*Tps proteins, excluding the putative N-terminal transit peptide regions, revealed that *Mt*Tps1 versus *Mt*Tps2 shared the highest identity (52.6%) and similarity (62.6%), followed by *Mt*Tps1 versus *Mt*Tps4, which shared 42.8% identity and 54.2% similarity (Table 1). *Mt*Tps2 and *Mt*Tps3 shared the lowest amino acid sequence identity (31%) and similarity (42.9%).

Enzymes Encoded by Small Gene Families

Probing *M. truncatula* genomic DNA with the selected cDNAs revealed that this species contains low copy numbers of the genes examined. Band-

TABLE 1. Amino Acid Sequence Identity (upper right) and Similarity (lower left) of *M. truncatula* Terpene Synthases Based on Overlapping Regions*

		% Identity			
		*Mt*Tps1	*Mt*Tps2	*Mt*Tps3	*Mt*Tps4
% Similarity	*Mt*Tps1	*	52.6	32.8	42.8
	*Mt*Tps2	62.6	*	31.0	37.5
	*Mt*Tps3	44.7	42.9	*	35.2
	*Mt*Tps4	54.2	49.9	47.5	*

*The putative N-terminal transit peptide sequences on *Mt*Tps3 and *Mt*Tps4 were excluded.

ing patterns indicate that each of the terpene synthase sequences are likely present in either a single copy (*MtTps*1, *MtTps*2, and *MtTps*3) or three copies (*MtTps*4) in the genome (Fig. 2). The *MtTps*2 cDNA sequence contains a single *Bam*HI recognition site and no *Eco*RI site, whereas the other clones contain neither a *Bam*HI nor an *Eco*RI recognition site. We have not obtained genomic sequences for these genes.

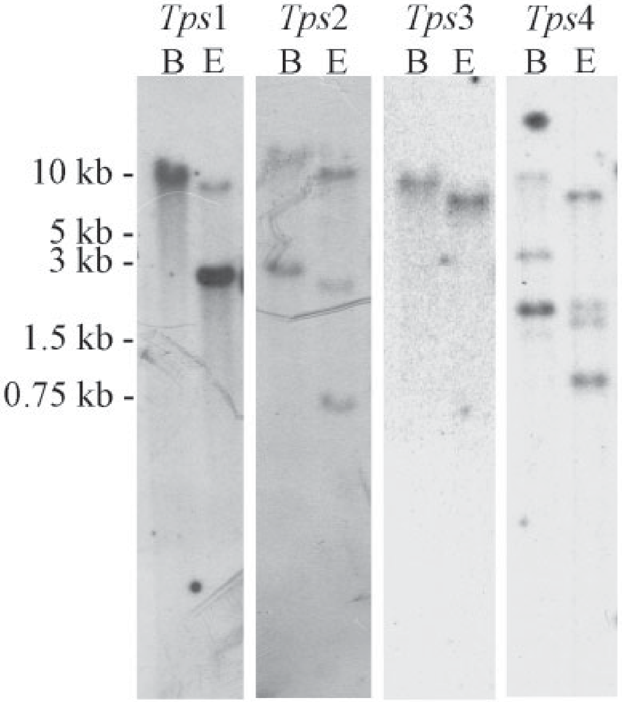

Fig. 2. *M. truncatula* DNA blots indicate low gene copy numbers of the sequences examined. Genomic DNA was digested with *Bam*HI (B) or *Eco*RI (E), separated via gel electrophoresis, transferred to nylon membranes and hybridized with the clone indicated. Relative location of DNA size markers is indicated at left.

Localization of *M. truncatula* Terpene Synthases

In vitro chloroplast import assays showed that *Mt*Tsp1 and *Mt*Tsp2 are not imported into isolated chloroplasts. This is the expected result if *MtTsp*1 and *MtTsp*2 encode cytosolic terpene synthases. However, *Mt*Tsp3 and *Mt*Tsp4 are imported into chloroplasts and are localized in the soluble fraction (Fig. 3), as would be expected for mono- and di-terpene synthases active in the latter stages of the plastid MEP pathway.

Wounding Induces Transcript Accumulation

Measurement of mRNA accumulation demonstrates that genes encoding terpene synthases were induced by mechanical- and insect-wounding. For all of the genes examined, the highest levels of transcripts were observed in leaves injured by insect herbivory (Fig. 4). For each gene, very low levels of RNA were present in undamaged leaves. Artificial damage also caused an increase in transcript accumulation, but to a lower degree than insect damage. Among the four genes, *MtTps*2 had the highest level of induction caused by mechanical damage, although not to the level observed after insect damage. On blots probed with *MtTps*2, two bands were consistently observed on RNA blots. This suggests there might be multiple forms of similar transcripts, derived from independent genes, that are cross-hybridizing on the membranes or that a single gene is subject to alternative splicing. For the *MtTps*2 transcripts, the two bands were always observed at similar levels, suggesting that if they are derived from independent genes, these genes must be coordinately regulated. Transcripts for each gene were also induced in systemic tissues of insect-damaged plants, although not to as high a level as in locally damaged leaves. The *MtTps*1 transcript was consistently the most strongly induced of all the genes examined. Insect herbivory is known to often elicit a greater plant response than artificial damage, probably due to the differing types of wounding or the presence of elicitor compounds associated with the insect (Korth and Dixon, 1997; Walker-Simmons et al., 1984).

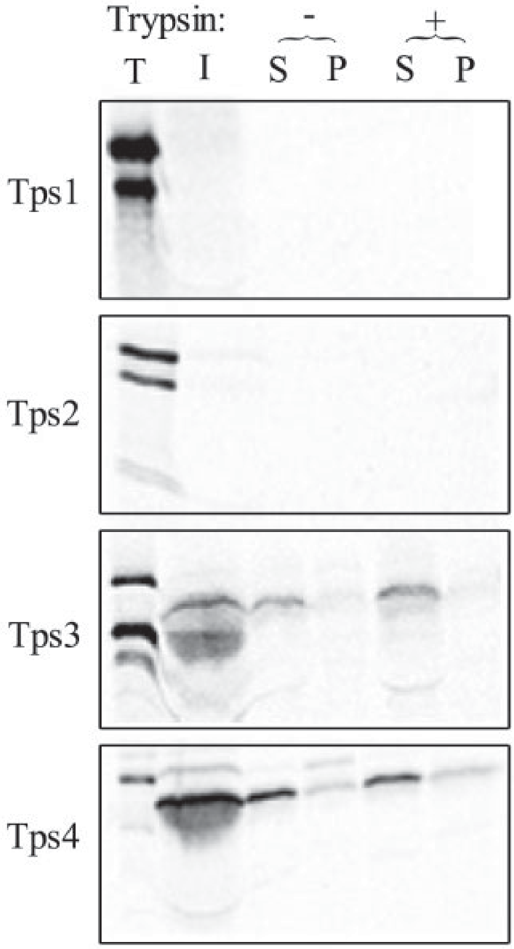

Fig. 3. *Mt*Tps3 and *Mt*Tps4, but not *Mt*Tps1 and *Mt*Tps2, are targeted to chloroplasts. Radiolabeled precursors of terpene synthases from *M. truncatula* were incubated with intact chloroplasts under import conditions. After the reaction, chloroplasts were re-isolated and directly subjected to SDS-PAGE analysis (I), or incubated with the import buffer without (–) or with (+) trypsin, lysed hypotonically, and fractionated into supernatant (S) and pellet (P) by centrifugation as described in Materials and Methods. T, 10% of the translation product subjected to the assay.

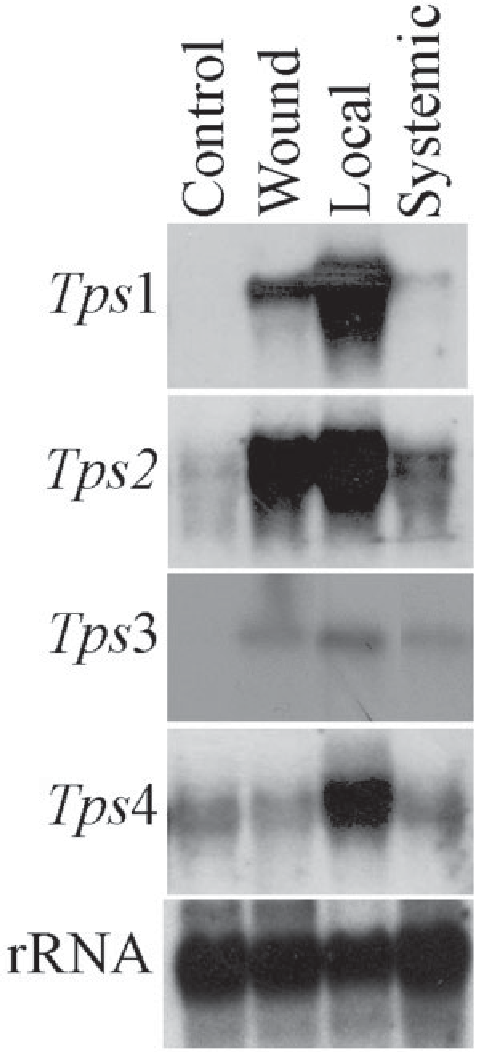

Fig. 4. Transcript accumulation as indicated by RNA blots, in leaves following artificial damage with tracing wheel ("wound"), or *S. exigua* herbivory. Locally wounded and systemic leaves from insect damaged plants were analyzed. "Control" samples are from undamaged leaves collected at the same time. Leaves were collected at 6 h after the initial damage. Membranes were hybridized with the indicated probes, and bands were visualized via autoradiography.

Transcript Induction by Methyl Jasmonate

Treatment of *M. truncatula* with volatile meJA led to transcript accumulation for each of the genes examined. Temporal transcript accumulation for each clone was measured at various time points between 30 min and 18 h after a 1-h exposure to low levels of volatile meJA (Fig. 5). For each of the clones, transcript levels clearly increased with time and returned to normal levels by 18 h after the initial exposure. As in wounding experiments, the *MtTps*1 transcripts were the most abundant among the clones tested. In addition, *MtTps*1 transcripts accumulated more rapidly than the other genes tested, with a visible signal on the RNA blot within 30 min and maximum transcript levels occurring 4 h after treatment. *MtTps*2 showed low levels of

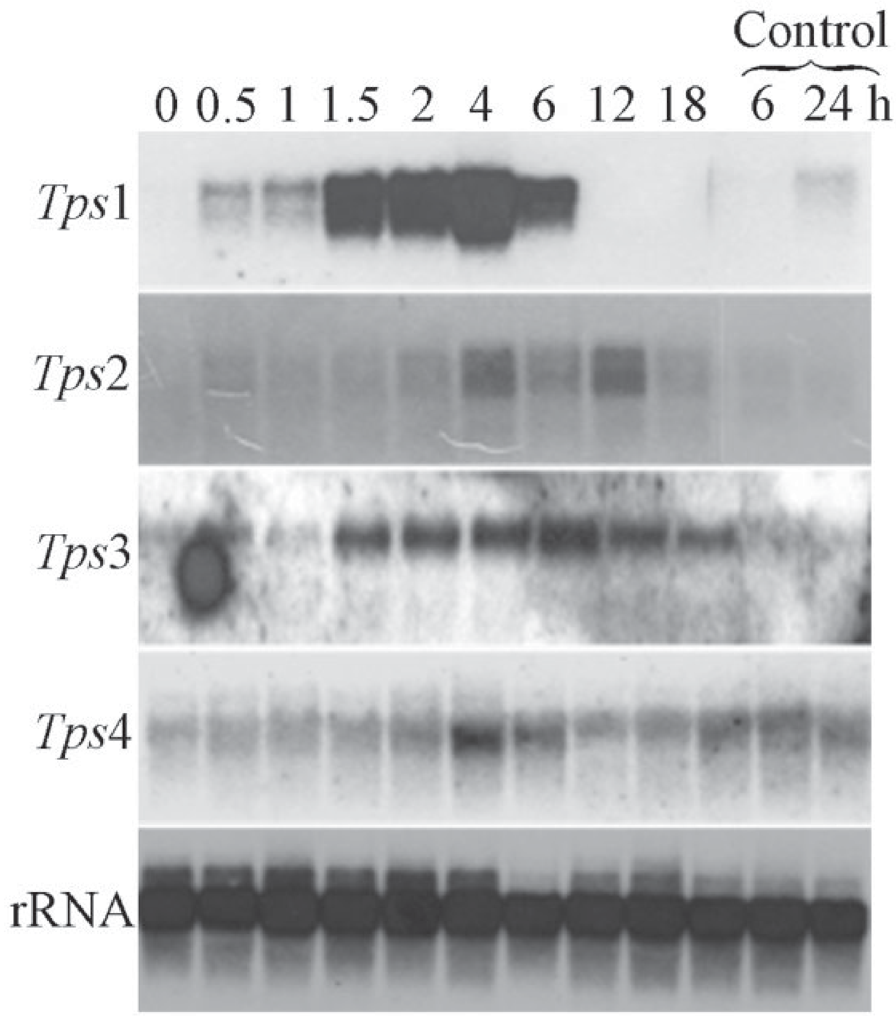

Fig. 5. Transcript accumulation of terpene synthase genes in response to treatment with volatile methyl jasmonate (meJA). Plants were exposed to 0.25 µM meJA for 1 h, and leaves were collected after treatment at times indicated. Control plants were treated the same as experimental plants, except that no meJA was added to the chambers. Membranes were hybridized with the indicated probes, and bands were visualized via autoradiography.

induction until 4–12 h after treatment, *MtTps*3 induction was more drawn out with abundant signals between 1.5–12 h after treatment, and *MtTps*4 was present at low levels, with the exception of 4 and 6 h after treatment (Fig. 5).

Transcript Induction by Lepidopteran Oral Factors

*MtTps*1 shows the greatest levels of accumulation and it responds rapidly to insect damage and meJA. Therefore, we focused our further efforts on the characterization of this gene. Transcript levels of *MtTps*1 increased in treatments with higher concentrations of *S. exigua* regurgitant (Fig. 6A). Low levels of *MtTps*1 transcript accumulate following mechanical damage, but transcript levels increase slightly even at a dilution of 1:500 of regurgitant. The data indicate that elicitors present in *S. exigua* regurgitant trigger responses in *M. truncatula* leading to the accumulation of cytosolic terpene synthase-encoding transcripts.

M. truncatula plants showed a response to two of the four fatty-acid:amino-acid conjugates (FACs) that are present in lepidopteran oral secretions (Fig. 6B). *MtTps*1 transcript levels were similar among buffer-treated leaves, 18:3-Gln-treated leaves, and

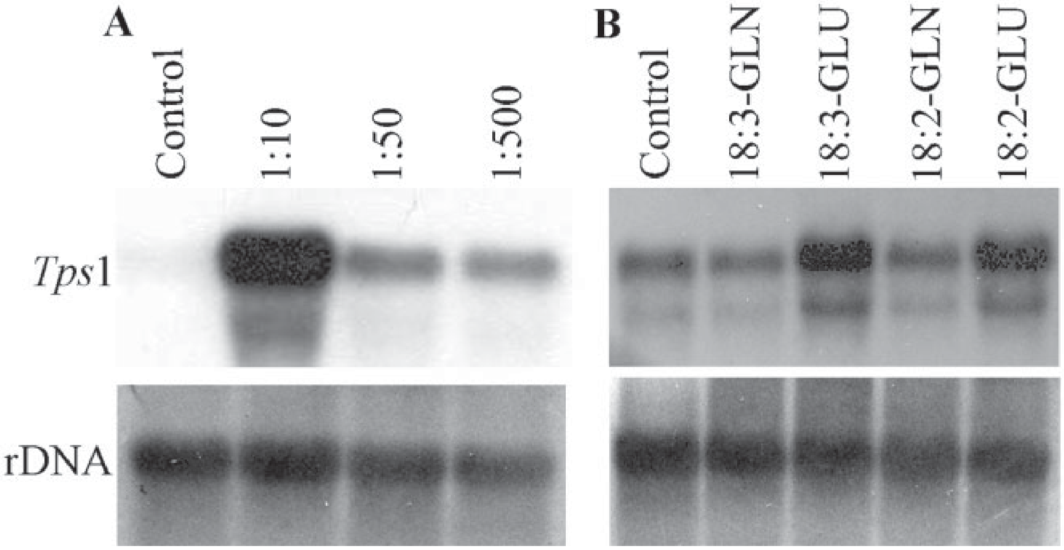

Fig. 6. Transcript accumulation as indicated by RNA blots, in wounded leaves following exposure to (**A**) buffer alone ("Control") or diluted *S. exigua* crude regurgitant, and (**B**) 80 ng/µL FAC elicitors identified as 18:3-GLN, 18:3-GLU, 18:2-GLN, and 18:2-GLU. Leaves were collected 6 h after application. Membranes were hybridized with radioisotope-labeled *MtTps*1 cDNA, and bands were visualized via autoradiography.

18:2-Gln-treated leaves. The elicitors 18:3-Glu and 18:2-Glu, factors not found in *S. exigua* regurgitant, triggered increased transcript accumulation of *MtTps*1.

DISCUSSION

Through analysis of the *M. truncatula* EST database, we were able to selectively isolate four cDNA clones that encode wound-inducible terpene synthases. Sequences of three of these clones (*MtTps*1, *MtTps*2, and *MtTps*4) are found only in the library originating from herbivore-damaged leaves. None of the four selected clones are found in the developing leaf library dataset, or in several other leaf-based libraries, suggesting that transcripts of these genes are not constitutively expressed in leaves. The validity of this "virtual screening" approach was confirmed via RNA blot analysis, which showed substantial accumulation of transcripts for the selected terpene synthase clones following insect damage.

The quantity of genomic copies of the genes analyzed here range from single-copy to three members of a small gene family. Transcripts for these genes accumulate to high levels following herbivory, and there seem to be limited genomic copies of each of the gene types. Because there are very few cross-hybridizing bands on genomic DNA blots, it is likely that clones *MtTps*1–4 encode insect-induced terpene synthases. These enzymes might play a direct role in biosynthesis of insect defense-associated terpenes in *M. truncatula*. Increased plant terpene synthase activities occur following herbivory and play a role in synthesis of induced terpenes (Bouwmeester, et al., 1999; Schnee, et al., 2002; Arimura, et al., 2004). *M. truncatula* releases volatile terpenes in response to herbivory (K. Korth, unpublished data). More definitive proof of the role of the clones described here will require determination of the terpene product(s) of each of the encoded enzymes in *M. truncatula*. Although the genes examined all encode conserved protein domains, it is well established that terpene synthases similar in sequence can differ greatly in terms of the specific products that they synthesize (Bohlmann et al., 1998). Furthermore, a single terpene synthase can produce several individual products (Dudareva et al., 2003; Schnee et al., 2002). Therefore, independent genes that cross-hybridize on DNA blots might encode enzymes with very different specificities. At the very least, the data indicate that there is a low degree of genetic redundancy for the sequences tested via genomic DNA blots and that *M. truncatula* can serve as a source of novel terpene synthases.

An aspartate-rich motif (DDxxD) and an arginine-tryptophan motif (RRx_8W) are characteristic of many terpene synthases (Bohlmann et al., 1998; Dudareva et al., 2003). Analysis of predicted peptide sequences indicates the DDxxD motif in each of the *M. truncatula* clones examined here. The RRx_8W was only present in *Mt*Tps1, at amino acid position R22. Bohlmann et al. (1998) have classified plant terpene synthases into at least six subfamilies (*Tspa-Tpsf*). *Mt*Tps3 is most similar to other monoterpene synthases and it lacks the RRx_8W motif, suggesting that it is a member of the recently proposed *Tpsg* subfamily (Dudareva et al., 2003).

Amino acid sequence analysis and subcellular localization suggest enzymatic roles for *Mt*Tps1-4. The *MtTps*1 and *MtTps*2 clones are most similar in sequence to previously characterized sesquiterpene synthases from other plant species. The proteins encoded by these genes are not imported into isolated chloroplasts (Fig. 3), an observation that supports the prediction of the products as cytosolic sesquiterpene synthases. Likewise, *Mt*Tps3 and *Mt*Tps4 are taken up by import-competent chloroplasts, in agreement with their sequence similarities with mono- or di-terpene synthases acting downstream of the plastid-localized MEP pathway.

Levels and timing of mRNA accumulation suggest that the *MtTps* clones are regulated at the transcriptional level in much the same way as other wound induced plant genes in a JA-mediated signaling pathway. For example, transcripts encoding proteinase inhibitors in tomato, *L. esculentum*, and alfalfa, *M. sativa*, accumulate to high levels following wounding or meJA treatment (Farmer et al., 1992). The induction of the *MtTps* genes suggests that the encoded proteins could play a critical role in the production of insect-induced volatile terpenes in *M. truncatula*. The release of jasmonate-induced volatile compounds has been observed in other plant species. Lima beans, *Phaseolus lunatus*, treated with JA, meJA, or spider mites release more volatile compounds than uninfested plants, which ultimately results in attraction of predatory mites (Dicke et al., 1999). A co-induction in terpene synthase transcript levels and volatile terpene release has been demonstrated (Schnee et al., 2002), including in legume species (Arimura et al., 2004).

Higher levels of *MtTps* transcripts accumulate in leaves after insect herbivory, as compared to mechanical damage, suggesting the activity of insect-derived elicitors or a unique type of damage during chewing by lepidopteran larvae. Treatment with *S. exigua* regurgitant triggers transcript accumulation in *M. truncatula* that is associated with the concentration of crude regurgitant. Herbivory has been shown to be mimicked by addition of crude insect regurgitant to a wound site, eliciting volatile release (Turlings et al., 1990). Analysis of *S. exigua* oral secretion showed that it contains volicitin, *N*-17-hydroxylinoleoyl-L-glutamine, free 17-hydroxylinolenic, glutamine conjugates of linolenic acid and linoleic acid, free linolenic and linoleic acids (Pohnert et al., 1999). Volicitin is also known to induce the release of volatiles in corn seedlings and it is as active as the crude regurgitant (Alborn et al., 2000).

It is interesting to note that in the tests with synthetic FAC factors, the highest transcript levels were found after application to *M. truncatula* of 18:3-GLU and 18:2-GLU, factors that are not found in *S. exigua* regurgitant (Pohnert et al., 1999; Alborn et al., 2000). Crude regurgitant applied to wound sites is a potent elicitor of *MtTps*1 transcript accumulation. Although 18:2-GLN and 18:3-GLN are reported to be found in *S. exigua*, they show no apparent activity as inducers of *MtTps*1 transcripts. Furthermore, among the synthetic FACs tested, the linolenic (18:3) conjugates are reported to have the highest volatile-inducing activities (Alborn et al., 2003). Although 18:3-GLU is a strong inducer of transcript accumulation in *M. truncatula*, 18:3-GLN is not. The particular blends of FACs, which vary between insect species, could have a major impact on the pattern of insect-induced gene expression that occurs in a plant (Voelckel and Baldwin, 2004).

The role of the *M. truncatula* terpene synthase gene products in defense is suggested by the strong and rapid induction of transcripts that occurred following insect herbivory. The enzymes that these genes encode, or genomic sequences that control their regulation, could provide valuable tools for further characterization of the plant response to chewing insects.

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