Tomato linalool synthase is induced in trichomes by jasmonic acid

Chris C. N. van Schie · Michel A. Haring · Robert C. Schuurink

Abstract Tomato (*Lycopersicon esculentum*) plants emit a blend of volatile organic compounds, which mainly consists of terpenes. Upon herbivory or wounding, the emission of several terpenes increases. We have identified and characterized the first two tomato monoterpene synthases, LeMTS1 and LeMTS2. Although these proteins were highly homologous, recombinant LeMTS1 protein produced (*R*)-linalool from geranyl diphosphate (GPP) and (*E*)-nerolidol from farnesyl diphosphate (FPP), while recombinant LeMTS2 produced *β*-phellandrene, *β*-myrcene, and sabinene from GPP. In addition, these genes were expressed in different tissues: *LeMTS1* was expressed in flowers, young leaves, stems, and petioles, while *LeMTS2* was strongest expressed in stems and roots. *LeMTS1* expression in leaves was induced by spider mite-infestation, wounding and jasmonic acid (JA)-treatment, while *LeMTS2* did not respond to these stimuli. The expression of *LeMTS1* in stems and petioles was predominantly detected in trichomes and could be induced by JA. Because JA treatment strongly induced emission of linalool and over-expression of *LeMTS1* in tomato resulted in increased production of linalool, we propose that LeMTS1 is a genuine linalool synthase. Our results underline the importance of trichomes in JA-induced terpene emission in tomato.

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Introduction

Plants produce a wide variety of terpenoids that have primary functions as hormones (gibberellins, abscisic acid, brassinolide), sterols, pigments (carotenoids, phytol), and as parts of electron carrier moieties (ubiquinone, plastoquinone) (McGarvey and Croteau 1995). Most terpenoids, however, are secondary metabolites: over 20,000 different terpenoid structures from plants have been described (Sacchettini and Poulter 1997). Some terpenoids function in the direct defence strategy of plants as phytoalexins, which accumulate upon pathogen infection. Sesquiterpene phytoalexins (e.g., capsidiol) are characteristic for the Solanaceae (Chappell and Nable 1987; Egea et al. 1996).

Volatile monoterpenes and sesquiterpenes can function in the interaction of plants with other organisms. They are present in floral scents that attract pollinators (Knudsen et al. 1993; Langenheim 1994) and are emitted by many plant species in response to herbivory by insects or spider mites. Induced terpene emission can attract predators or parasitoids of the herbivores, a mechanism commonly referred to as the indirect defense strategy of plants. This has been studied in, for instance, Arabidopsis (*Arabidopsis thaliana*) (Van Poecke et al. 2001), lima bean (*Phaseolus lunatus*) (Dicke 1994; Takabayashi and Dicke 1996), tobacco (*Nicotiana tabacum*; *N. attenuata*), maize (*Zea mays*), and cotton (*Gossypium hirsutum*) (De Moraes et al.

1998; Pare and Tumlinson 1999), and tomato (Kant et al. 2004; Takabayashi et al. 2000).

These volatile terpenes are produced by sesqui- and monoterpene synthases of which several are induced by herbivory. For instance, nerolidol synthase activity is induced in lima bean, cucumber (*Cucumis sativus*), and maize (Bouwmeester et al. 1999; Degenhardt and Gershenzon 2000), whereas transcript levels of various other sesquiterpene synthases increase in, amongst others, maize (Shen et al. 2000), cucumber (Mercke et al. 2004), and wormwood (*Artemisia annua*) (Cai et al. 2002). In Arabidopsis, caterpillars (*Pieris rapae*) induce the myrcene/ocimene synthase *AtTPS10* and the β-ocimene synthase *AtTPS03*, which coincides with increased myrcene emission (Van Poecke et al. 2001). Moreover, a β-ocimene synthase (*LjEβOS*) has been identified from Lotus (*Lotus japonicus*), which is induced by spider mite-feeding, resulting in increased emission of β-ocimene (Arimura et al. 2004b). However, artificial wounding or jasmonic acid (JA) treatment are often also sufficient to induce terpene synthases. The wound- and JA-induced synthesis of terpenes by coniferous plants is well described (Martin et al. 2002; Miller et al. 2005; Steele et al. 1995), and these treatments also mimic herbivore-induced responses in several angiosperms (Arimura et al. 2004a; Gomez et al. 2005; Schnee et al. 2002; Shen et al. 2000).

Here we describe the identification and characterization of the first two monoterpene synthases from a solanaceous species (tomato), *LeMTS1* and *LeMTS2*. We provide evidence that these genes are differentially regulated and more importantly, that expression of linalool synthase *LeMTS1* is restricted to trichomes and induced by JA.

Results

Identification of tomato terpene synthases 1 and 2 (*LeMTS1* and *LeMTS2*)

In order to identify tomato monoterpene synthases that are induced upon spider mite herbivory, we first queried tomato EST databases (www.tigr.org and www.ncbi.nlm.nih.gov/BLAST/) with known monoterpene synthase sequences from other plant species. Retrieved tomato EST sequences were used to design a primer specific for monoterpene synthases (Fig. 1). We then used cDNA derived from spider mite-infested plants, to amplify a single 850 bp fragment with this primer and an oligo dT(18) primer. This fragment was subsequently used as a probe to screen available tomato cDNA libraries. Two different full-length cDNAs were identified; *LeMTS1* (AY840091) was isolated from tomato leaves (sequence identical to the probe) and *LeMTS2* (AY840092) from

tomato roots and stems. The *LeMTS1* and *LeMTS2* open reading frames encode proteins of 609 and 590 amino acids (Fig. 1), which have predicted masses of 70.8 and 68.6 kDa, respectively, and are 71% identical. LeMTS1 and LeMTS2 contain amino acid motifs conserved in angiosperm (mono)terpene synthases. These are, based on LeMTS1 amino acid positions: R(45)R(46)W(55), required for the initial diphosphate migration (Williams et al. 1998); D(362)D(363)D(366), required for binding of a Mg^{2+} or Mn^{2+} ion, which is used as cofactor (Cane et al. 1996; Starks et al. 1997); D(505)T(509)E(513), required for binding of a second Mg^{2+} or Mn^{2+} cofactor; and D(586) in the active site cleft (Schwab et al. 2001; Whittington et al. 2002). LeMTS1 and LeMTS2 contain both putative (predicted with TargetP; Emanuelsson et al. 2000) plastid targeting signal peptides, which should be processed in the region upstream of the conserved R(45)R(46) arginine pair.

Remarkably, LeMTS1 contains an extra sequence stretch of 22 amino acids starting at N170 (Fig. 1). This sequence is not found in any other terpene synthase, nor does it have homology to any protein in the NCBI database (www.ncbi.nlm.nih.gov/BLAST/). Its presence can also not be explained by alternative splicing since the flanks do not contain canonical splice sites. This extra internal element is not an artefact of the isolated cDNA since it was present in four independent cDNAs isolated from different organs (data not shown).

LeMTS1 and LeMTS2 belong to the TPSb subfamily of terpene synthases

LeMTS1 and LeMTS2 are more similar to typical monoterpene synthases from, e.g., mint and citrus than to a typical tomato sesquiterpene synthase (Fig. 1). Based on protein sequence relatedness, LeMTS1 and LeMTS2 can be classified in the *TPSb* subfamily (Fig. 2), a distinct group of angiosperm monoterpene synthases, according to the original classification proposed by Bohlmann et al. (1997). So, unlike the snapdragon (*Antirrhinum majus*) monoterpene synthases from subfamily TPSg (Dudareva et al. 2003), including an Arabidopsis and a strawberry terpene synthase, the monoterpene synthases from the solanaceous family do not seem to form a new subfamily.

Terpene synthase activity of recombinant LeMTS1 and LeMTS2

Recombinant LeMTS1 and LeMTS2 proteins were produced in *Escherichia coli* in order to characterize their enzymatic activities. The plastid targeting peptide was deleted up to one amino acid upstream of the RRX_8W motif (Fig. 1) to produce a His-tagged 'pseudo-mature' form of the proteins, since it has been reported that this can
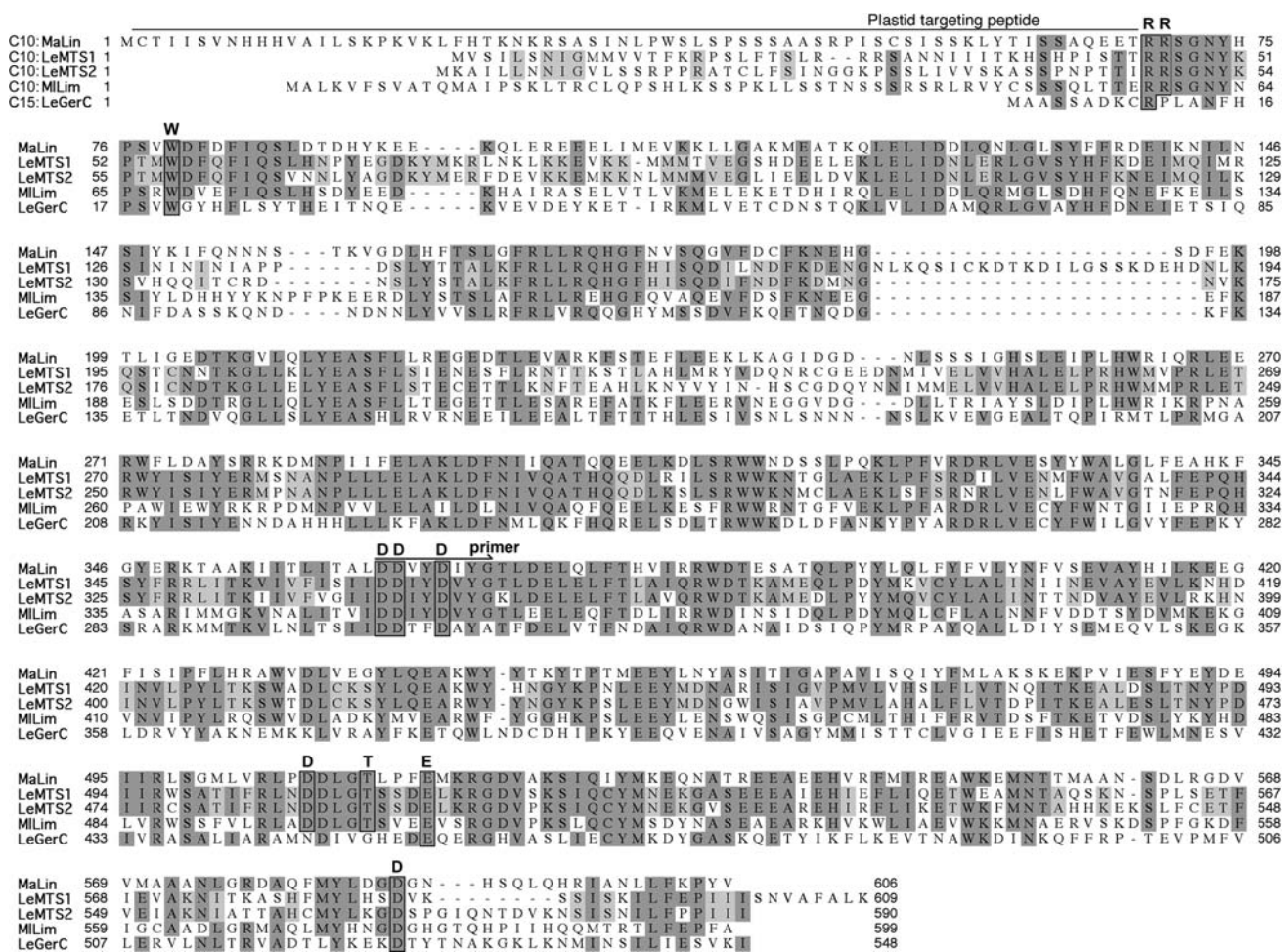
Fig. 1 Alignment of deduced amino acid sequences of LeMTS1 and LeMTS2 with three terpene synthases. A ClustalW alignment is shown of Mentha aquatica linalool synthase, a monoterpene synthase (C10), MaLin (AAL99381); Lycopersicon esculentum monoterpene synthase 1, LeMTS1 (AY840091); *L. esculentum* monoterpene synthase 2, LeMTS2 (AY840092); Mentha longifolia limonene synthase, MlLim (AAD50304), and *L. esculentum* germacrene C synthase, a typical sesquiterpene synthase (C15), LeGerC (AAC39432). Amino acids are shaded dark if identical in at least three of the five sequences and light if identical in LeMTS1 and LeMTS2 only. Conserved amino acid motifs typical for terpene synthases are boxed: RRx_8W (Williams et al. 1998), DDxxD (Cane et al. 1996; Starks et al. 1997), Dx_3Tx_3E, and D (Schwab et al. 2001; Whittington et al. 2002). The presence of putative N-terminal plastid targeting peptides in the four monoterpene synthases and the primer used for amplifying the initial LeMTS fragment from cDNA of spider mite-infested leaves are indicated

improve protein expression without affecting activity and product specificity of the enzymes (Bohlmann et al. 1999; Williams et al. 1998). The soluble protein fractions were assayed for terpene synthase activity and products were analyzed on a GC coupled to a Time Of Flight-MS. Using the monoterpene precursor geranyl diphosphate (GPP) as substrate, LeMTS1 generated only linalool whereas LeMTS2 generated several monoterpene products: β-phellandrene, β-myrcene, and sabinene (Fig. 3a). Mass spectra and relative retention indices (Adams 2001) or authentic standards confirmed the identification of terpene reaction products. Extracts of *E. coli* cells expressing the empty vector control generated minor amounts of linalool (Fig. 3a) and occasionally geraniol. Thermally induced autoconversion or solvolysis of GPP to linalool has been

observed before (Crowell et al. 2002; Jia et al. 1999), as well as conversion to geraniol by aspecific hydrolase or phosphatase activity in protein extracts (Crowell et al. 2002). However, LeMTS1 product levels are several hundred-fold higher than the background levels of the control (Fig. 3a).

Enantiomer separation on an enantiomer-selective column showed that LeMTS1 produced only (R)-linalool (data not shown). LeMTS1 was also able to utilize the sesquiterpene precursor farnesyl diphosphate (FPP) as substrate to produce (E)-nerolidol (Fig. 3b), whereas LeMTS2 had no detectable sesquiterpene synthase activity. Both enzymes were unable to form terpene products from the diterpene precursor geranylgeranyl diphosphate.
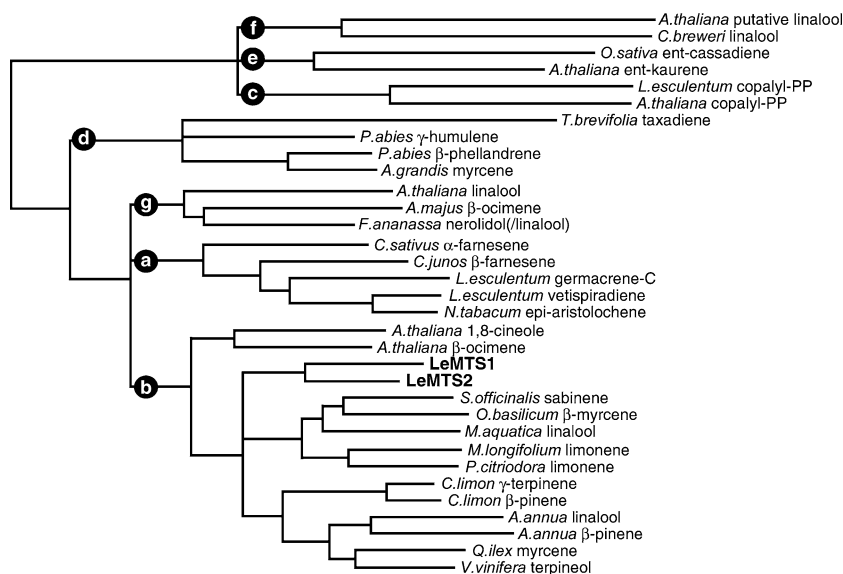
Fig. 2 LeMTS1 and LeMTS2 belong to the TPSb subfamily of terpene synthases. The TPS family is divided into subfamilies according to the nomenclature of Bohlmann et al. (1997). TPSa: angiosperm sesquiterpene synthases, TPSb: angiosperm monoterpene synthases, TPSc: angiosperm diterpene synthases, TPSd: gymnosperm mono-sesqui- and diterpene synthases, TPSe: a second group of angiosperm diterpene synthases, TPSf: a distinct group of (putative) linalool synthases mainly from Clarkia (Dudareva et al. 1996) and TPSg: a recently identified group of monoterpene synthases mainly from Antirrhinum (Dudareva et al. 2003). A limited amount of representative terpene synthases is shown. The bootstrapped phylo-genetic tree was constructed using PAUP and TreeView software. A representative tree of 100 replicates is shown. Genbank protein accessions of terpene synthases shown are, top to bottom: TPSf: AAL24105, AAC49395; TPSe: BAC56714, AAC39443; TPSc: BAA84918, NP192187; TPSd: AAK83566, AAK39129, AAK39127, AAB71084; TPSg: NP176361, AAO42614, CAD57081; TPSa: AAU05951, AAK54279, AAC39432, AAG09949, Q40577; TPSb: NP189212, NP567511, AY840091, AY840092, AAC26018, AAV63791, AAL99381, AAD50304, AAG31435, AAM53943, AAM53945, AAF13356, AAK58723, CAC41012, AAS79351

Fig. 3 Enzymatic activity of recombinant LeMTS1 and LeMTS2 proteins. (**A**) GC/MS chromatograms of the LeMTS1 and LeMTS2 monoterpene products and the empty vector control with GPP as substrate. (**B**) GC/MS chromatogram of the LeMTS1 sesquiterpene product and the empty vector control with FPP as substrate. The chromatograms show detector responses for the terpene-specific ion mass 93. Products were identified as linalool (1), sabinene (2), *β*-myrcene (3), *β*-phellandrene (4), and (*E*)-nerolidol (5). Representative chromatograms are shown from assays of at least two independent experiments
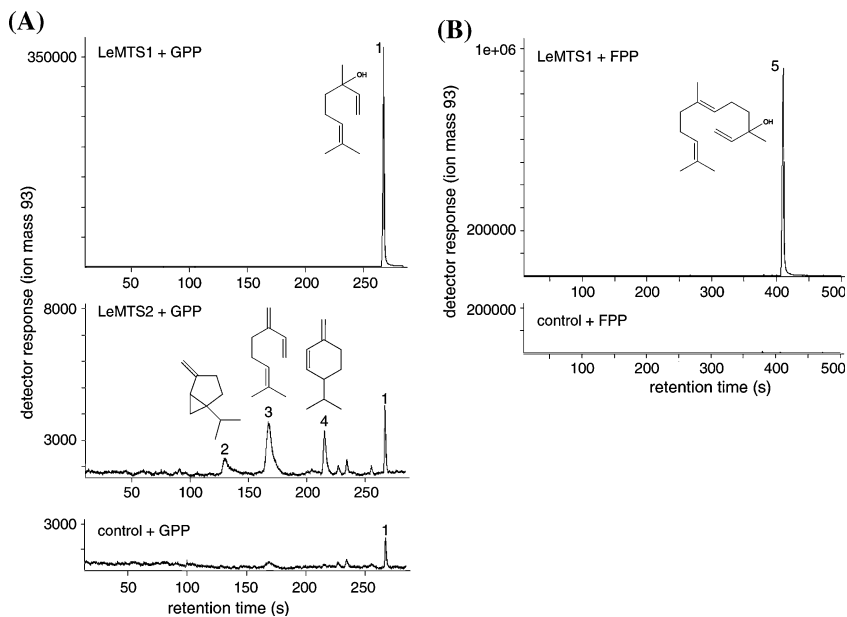
LeMTS1 and *LeMTS2* are differentially expressed in various plant organs

We investigated the organ specificity of *LeMTS1* and *LeMTS2* expression in a mature (13 weeks old) tomato plant (Fig. 4a). Transcripts of *LeMTS1* were detected in young fruit, flower buds, petals, sepals, stems, petioles, and in young leaves. The expression pattern of *LeMTS2* was very different: no transcripts were detected in petals, sepals, and leaves. However, *LeMTS2* was expressed in roots.
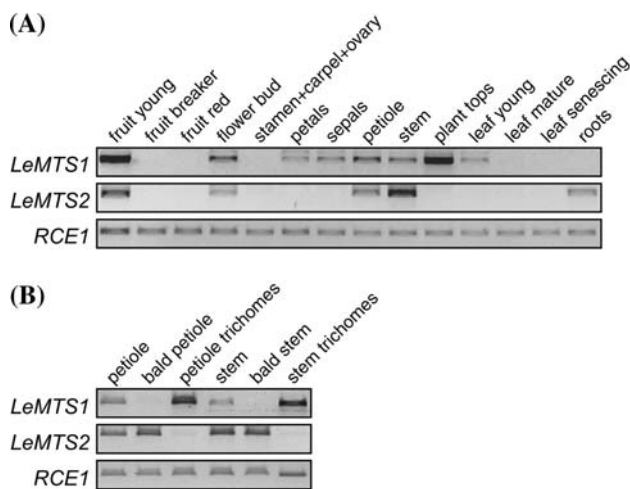
Fig. 4 Spatial expression patterns of *LeMTS1* and *LeMTS2* in tomato. (**A**) Expression of *LeMTS1* and *LeMTS2* in different organs of an adult tomato plant. (**B**) Expression in trichomes or complementary tissue of petioles and stems. Expression was analyzed by RT-PCR. The data from one of two independent tissue sets that gave similar results is shown. RUB1 conjugating enzyme (*RCE1*, www.tigr.org: TC153679) was used as constitutive control. Ethidium bromide-stained agarose gels are shown

For plants that contain glandular trichomes, monoterpene production is considered to be localized exclusively in these organs (Gershenzon et al. 1992; Iijima et al. 2004; Kutchan 2005; Turner et al. 1999; Turner and Croteau 2004). Tomato plants possess several types of trichomes including glandular trichomes that are present in high density on leaves, petioles, and stems and that contain terpenes (Maluf et al. 2001; Snyder and Carter 1985). Therefore, we investigated the location of *LeMTS1* and *LeMTS2* expression in trichomes of petioles and stems. This showed that *LeMTS1* is expressed in trichomes, whereas *LeMTS2* is expressed in the complementary tissue (Fig. 4b).

LeMTS1 but not *LeMTS2* is induced by spider mite feeding, artificial wounding, and jasmonic acid

To study whether *LeMTS1* and *LeMTS2* were induced upon spider mite feeding, changes in transcript levels of intact plants were analyzed by RT-PCR since *LeMTS* gene-expression was too low to determine by RNA gel-blot analysis. Expression of *LeMTS1* was indeed induced in leaves by spider mite-feeding, but *LeMTS2*, which expression was very low in leaves, was not (Fig. 5a). Spider mites damage plant tissue and induce JA- and SA-responsive genes such as a wound-induced proteinase inhibitor (*WIPI-II*) and a pathogenesis related protein (*PRP6*), respectively (Kant et al. 2004). Therefore, we investigated the effects of artificial wounding and exogenous application of JA and SA (Fig. 5a). Both artificial wounding and JA induced *LeMTS1*. In contrast, *LeMTS2*
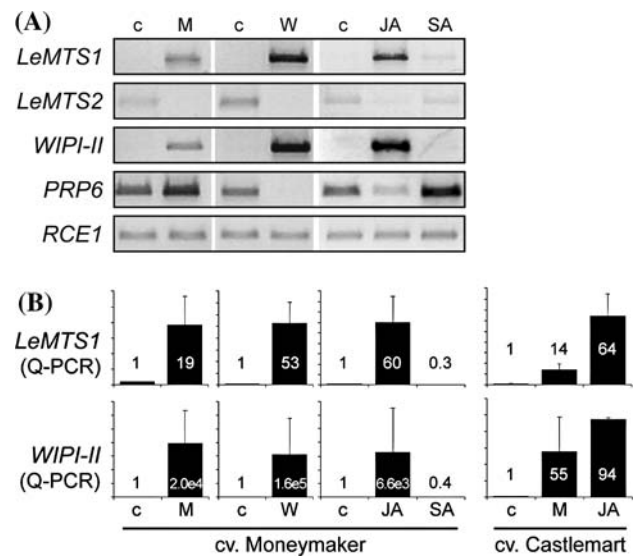

Fig. 5 Induction of *LeMTS1* in tomato leaves in response to spider mites, wounding, and jasmonic acid (JA). (**A**) Gene expression analysis by semi-quantitative RT-PCR of *LeMTS1* and *LeMTS2* after various treatments, compared to the well known JA- and SA-marker genes *WIPI-II* (Graham et al. 1985) and *PRP6* (van Kan et al. 1992), respectively. Treatments: JA, SA, and artificial wounding (W) after 24 h and spider mite-infestation (M) after 48 h. A representative result from three independent experiments is shown. RUB1 conjugating enzyme (*RCE1*) was used as constitutive control. Ethidium bromide-stained agarose gels are shown. (**B**) Validation of *LeMTS1* and *WIPI-II* induction by real-time Q-RT-PCR. Two different tomato cultivars were used to verify induction by mites and JA. Values represent expression levels relative to controls, calculated after correction for expression of the control gene *RCE1* (TC153679, www.tigr.org). Average values and standard errors are shown from three to five independent replicates

was not induced by these treatments (Fig. 5a). SA-treatment induced the SA-marker *PRP6* but neither *LeMTS1* nor *LeMTS2* was increased. Induction of *LeMTS1* and *WIPI-II* was validated by real-time Q-RT-PCR. This showed consistent and clear induction of both genes by JA, artificial wounding and spider mites, although there was large variation in the level of induction between independent experiments (Fig. 5b). We routinely used the tomato cultivar Moneymaker for our experiments. However, in our earlier work (Ament et al. 2004; Kant et al. 2004) the cultivar Castlemart (CM) was used because it has the same genetic background as the *defenseless1* (*def-1*) mutant (Howe et al. 1996). Here, we show that spider mite- and JA-induced *LeMTS1* expression occurred both in CM and Moneymaker (Fig. 5b).

To investigate whether spider mite-induced expression of *LeMTS1* was dependent on JA or SA we made use of the *def-1* mutant, which is impaired in induced JA-accumulation (Howe et al. 1996) and the SA-deficient, transgenic *NahG* tomato line (Brading et al. 2000). In plants with the *def-1* mutation, *LeMTS1* was not induced by spider mites,
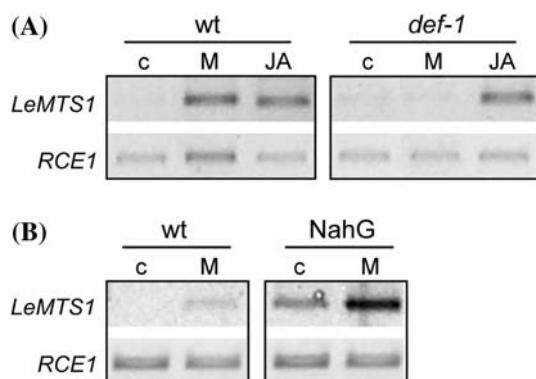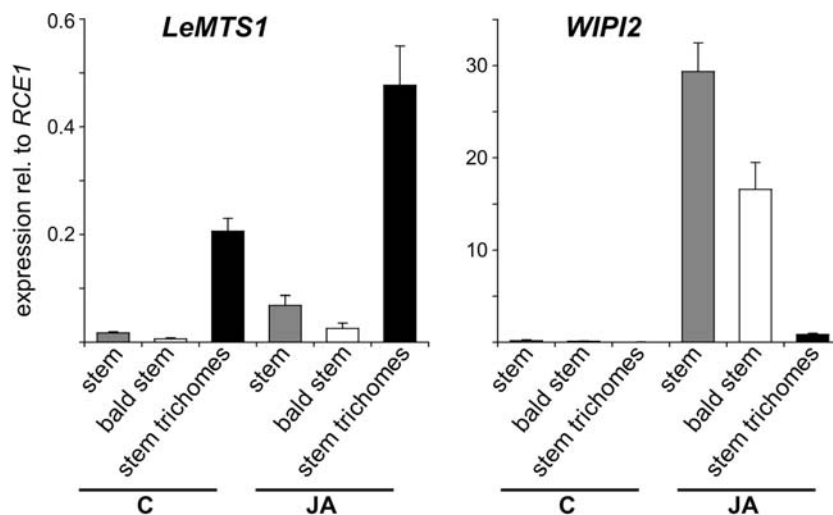
Fig. 6 Spider mite-induced *LeMTS1* expression is dependent on jasmonic acid (*JA*). (**A**) Expression of *LeMTS1* in wild type (*wt*) or JA deficient *def-1* mutant plants infested with spider mites (M) or treated with JA for 2 days. (**B**) *LeMTS1* expression in wild type and SA deficient *NahG* plants in response to 2 days of spider mite-feeding. Gene expression was analyzed by RT-PCR, a representative result from one out of two independent experiments is shown. RUB1 conjugating enzyme (*RCE1*) was used as constitutive control. Ethidium bromide-stained agarose gels are shown

whereas the expression could be induced by exogenous JA (Fig. 6a). In contrast, the *NahG* plants showed higher and clearly induced *LeMTS1* expression (Fig. 6b). This shows that induction of *LeMTS1* expression is dependent on JA but not on SA.

JA induces expression of *LeMTS1* in trichomes

Since basal LeMTS1 expression in stems and petioles seemed to be restricted to trichomes (Fig. 4b), we addressed the question whether JA would induce expression of LeMTS1 solely in trichomes or also in other tissues. Indeed, JA-induced LeMTS1 expression was

strongest in trichomes (Fig. 7). Interestingly, WIPI-II expression showed the opposite pattern. WIPI-II expression is very low in trichomes and basal as well as strongly induced WIPI-II expression occurred mainly in the complementary stem tissues.

Linalool accumulates in glandular trichomes and linalool emission is induced by wounding, jasmonic acid, and by ectopic *LeMTS1* overexpression

Figure 4b showed that, in petioles and stems, *LeMTS1* was expressed specifically in the trichomes. Extraction of terpenes from the same tissues of that experiment demonstrated that linalool was produced predominantly in trichomes (Fig. 8). (*E*)-nerolidol and *β*-phellandrene also accumulated in trichomes. Conversely, the volatile benzenoid methyl-salicylate was not produced in trichomes and was also clearly detected in roots, in contrast to most terpenes.

Because *LeMTS1* was induced by spider mite-infestation, wounding and JA, we investigated whether the emission of its in vitro products was induced concomitantly. Analysis of volatile production during 2 days of wounding or JA-treatment, conditions that induce *LeMTS1* expression (Fig. 5), revealed that plants emitted more linalool after both treatments (Fig. 9a), while nerolidol emission was not induced.

Next, we tried to overexpress the full length *LeMTS1* cDNA (including the putative targeting peptide) in tomato plants, under control of the CaMV 35S promoter. Four transformation experiments yielded only two transformants of which one overexpressed *LeMTS1*. Compared to empty vector-transformed plants, *LeMTS1* overexpressing plants emitted several hundred-fold more linalool into the plants headspace (Fig. 9b), while emis-

Fig. 7 LeMTS1 is induced in trichomes by JA. Expression analysis of *LeMTS1* and *WIPI-II* by real-time Q-RT-PCR. Intact plants were treated with JA, and subsequently trichomes were separated from remaining tissues of stems. The results from four independent experiments are shown as mean values and standard errors. Expression levels are indicated as expression relative to the control gene *RCE1*
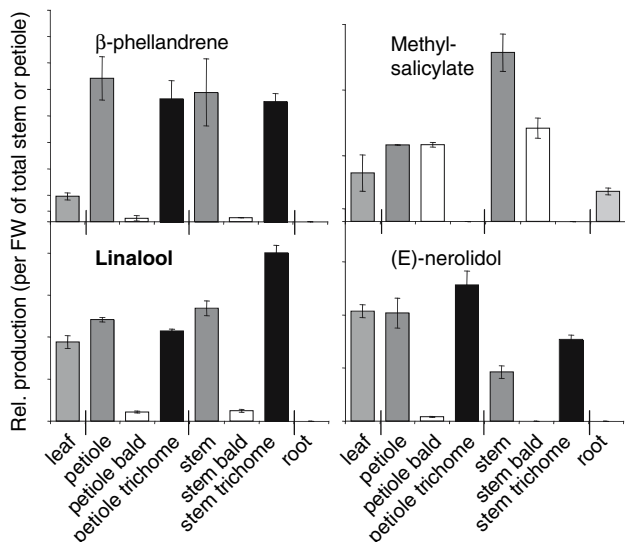
Fig. 8 Linalool accumulation in trichomes. Relative levels of linalool, a selection of other terpenes and methyl salicylate in extracts of petioles and stems before and after removal of trichomes compared to total leaf and root extracts. Equal amounts of the different tissues were used (freshweight, 0.5 g), and trichome material was corrected for freshweight of the original petiole or stem. Averages of two measurements and min/max are shown

sion of other terpenes, including nerolidol, did not differ significantly from control plants. This suggests that LeMTS1 functions in planta as a true linalool synthase.

Discussion

Monoterpenes are abundantly emitted by tomato plants. For instance β-pinene, β-myrcene, 2-carene, β-phellandrene, limonene, and terpinolene are constitutively emitted, while linalool and β-ocimene emission is induced after spider mite feeding (Kant et al. 2004). In this paper, we describe the first two monoterpene synthases from tomato, *LeMTS1* and *LeMTS2* and show that *LeMTS1* is induced by JA in trichomes.

Identification of two tomato monoterpene synthases

In vitro assays with the recombinant LeMTS1 and LeMTS2 proteins show that LeMTS1 has both (R)-linalool synthase and (E)-nerolidol synthase activity, and that LeMTS2 is a β-phellandrene/β-myrcene/sabinene synthase (Fig. 2a). LeMTS1 activity in planta seems restricted to linalool synthase activity since increased *LeMTS1* expression by wounding, JA-treatment and ectopic overexpression led to increased linalool emission but did not affect nerolidol levels (Figs. 5, 9).

Generally speaking, monoterpene synthases are plastid targeted and use GPP, while sesquiterpene synthases use FPP in the cytosol. There are however some exceptions to this rule. For instance, a cytosolic strawberry nerolidol
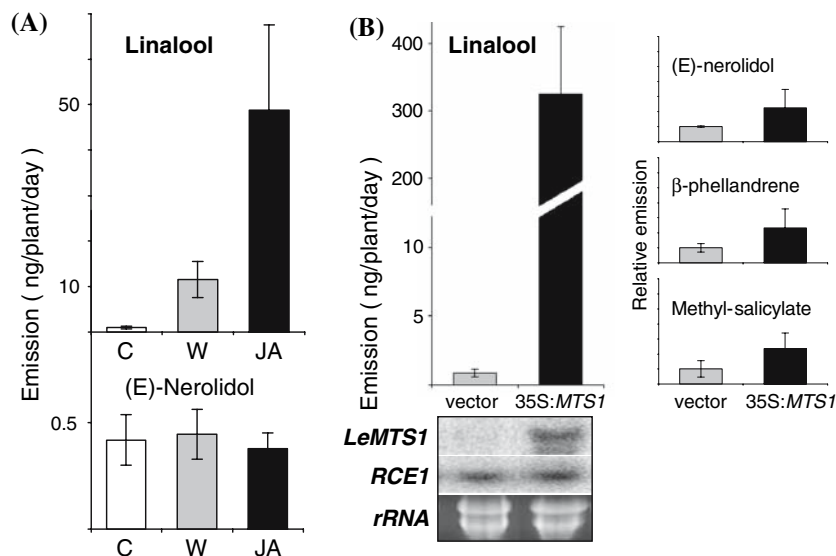

Fig. 9 Increased linalool emission. (**A**) Linalool and (E)-nerolidol emission by intact tomato plants that were wounded (W) or treated with 1 mM jasmonic acid (JA) twice a day, for 2 days. The bar-graph shows the average (n = 3) emission over 2 days compared to the control and standard errors. Data (corrected for unequal variances) were analyzed by means of ANOVA followed by a Fishers-LSD post hoc-test. Linalool emission for both JA and wounding treatments was significantly increased compared to the control (P < 0.05), whereas (E)-nerolidol emission was not. (**B**) Linalool emission by a transgenic tomato line overexpressing LeMTS1. Headspaces were sampled from desiccators containing two plants each and compared to that of plants transformed with the empty vector. The graph expresses average values and standard deviations of four measurements. Emission of (E)-nerolidol, β-phellandrene, and methyl-salicylate did not significantly differ between plants. LeMTS1 overexpression in leaves was confirmed by RNA gel-blot analysis. The expression of RUB1 conjugating enzyme (RCE1) and ribosomal RNA levels (ethidium bromide-stained gel) are shown to indicate equal loading of the gel

synthase (FaNES1) has both (3S)(E)-nerolidol synthase and S-linalool synthase activity and is likely responsible for both linalool and nerolidol synthesis in planta (Aharoni et al. 2004). Vice versa, when FaNES1 was targeted to the plastid in Arabidopsis it also produced both linalool and nerolidol (Aharoni et al. 2003). The authors suggested that GPP and FPP substrate pools are not strictly separated between the cytosol and plastid, which has recently been supported by similar engineering experiments in tobacco (Wu et al. 2006). These observations might be explainable by for instance cytosolic as well as plastidial targeting of GPP synthase (GPS) in Arabidopsis and *Litospermum erythrorizon* (Bouvier et al. 2000; Sommer et al. 1995). In addition, FPP might be transported from the cytosol into plastids (Lichtenthaler 1999) and finally, the prenyltransferases GPS, FPS or GGPS might generate multiple products. However, since induced *LeMTS1* expression does not correlate with increased nerolidol emission but only with increased linalool emission (Figs. 5, 9), we consider LeMTS1 to be a linalool synthase. Furthermore, we hypothesize that the plastidial FPP concentrations in tomato are negligible.

Despite their high sequence homology (Fig. 1), LeMTS1 and LeMTS2 have different biochemical activities. It is commonly found that different terpene synthases within one plant species often have higher sequence similarity to one another than to functionally related terpene synthases of other plant species. For example, β-pinene synthase from citrus (*Citrus limon*) is more homologous to the citrus γ-terpinene synthase than to β-pinene synthase from wormwood (Fig. 2). It is also known from work with other terpene synthases that product specificity can depend on only a few amino acids (Kollner et al. 2004; Rising et al. 2000; Yoshikuni et al. 2006) and thus, product specificity of terpene synthases is not predictable on basis of protein sequence or specific amino acid residues. By comparing terpene synthase structures, attempts have been made to ascribe substrate binding and substrate modifications to certain residues. Modeling of the active sites of a Salvia (*Salvia officinalis*) sabinene synthase, a mint (*Mentha citrata*) limonene synthase and a mint linalool synthase led to the following observation (Crowell et al. 2002): compared to the other two enzymes, the linalool synthase has a three amino acid deletion in the active site pocket (two amino acids after the conserved D591, see Fig. 1), resulting in a more open structure of the so called J/K loop and providing an easier access of water during substrate ionization. Water access results in the premature release of the intermediate monoterpenol carbo-cation, before cyclization to a monoterpene olefin (non-hydrated) can occur. The tomato linalool synthase LeMTS1 also has a deletion in the active site pocket after the conserved D586 (Fig. 1). Although the deletion is eight instead of three amino acids, it indicates

that LeMTS1 might utilize a similar structural mechanism to generate a monoterpene alcohol.

LeMTS1 expression correlates with induced linalool emission

The induction of *LeMTS1* expression correlated with the increased emission of linalool by tomato plants. Wounding and JA application induced *LeMTS1* (Fig. 5) as well as linalool emission (Fig. 9a). Induction of *LeMTS1* upon spider mite-feeding (Fig. 5) coincided with linalool emission (Kant et al. 2004) in the tomato cultivar CM. Moreover, overexpression of *LeMTS1* resulted in enhanced production of linalool (Fig. 9b) and no other volatile terpenes, providing evidence for in planta linalool synthase activity of LeMTS1. RNAi lines suppressing *LeMTS1* expression in trichomes would provide additional evidence that LeMTS1 acts as a linalool synthase in planta.

It is generally known that successful overexpression of terpene synthases can be problematic (reviewed by Chappell 2004). Substrate limitation and product conversions can explain low-terpene production levels (Aharoni et al. 2003; Lucker et al. 2001; Ohara et al. 2003). In addition, ectopic terpene synthase overexpression might lead to product toxicity (Aharoni et al. 2006; Besumbes et al. 2004) and selection for plant lines with low transgene expression or low terpene accumulation (Diemer et al. 2001; Krasnyanski et al. 1999). We managed to regenerate only one *LeMTS1* overexpressing plant line after four successive, independent transformations. The empty vector and unrelated constructs gave at least 20-fold higher transformation frequencies. Similarly, *LeMTS2* overexpression was unsuccessful, seven transgenic plant lines showed only expression of truncated transcripts (data not shown).

LeMTS2 is expressed in roots, stems and petioles (Fig. 4a). In roots, only minute amounts of the main LeMTS2 product β-phellandrene were detected, hardly visible in Fig. 8 since levels are ~300-fold lower than in leaves. The role of monoterpenes in roots remains unclear, although they might play a role in the defense response of plants. The release of the monoterpene 1,8-cineole from Arabidopsis roots is induced upon bacterial pathogen infection (Steeghs et al. 2004), although transcriptional regulation of the corresponding 1,8-cineole synthase (Chen et al. 2004) has not been shown yet.

Although *LeMTS2* is hardly expressed in tomato leaves (Fig. 4a), β-phellandrene and β-myrcene are constitutively emitted by tomato plants (Buttery et al. 1987; Kant et al. 2004). This suggests that the constitutive low expression of *LeMTS2* might be sufficient to generate these monoterpenes. However, like the majority of volatile terpenes (data not shown), β-phellandrene is mostly located in trichomes

(Fig. 8) where *LeMTS2* is not expressed (Fig. 4b). This indicates that there are other β-phellandrene/β-myrcene synthases in tomato that synthesize these terpenes.

JA-induced *LeMTS1* expression occurs in trichomes

Although the presence of terpene synthases in trichomes has been well documented (Bertea et al. 2006; Iijima et al. 2004; Lange et al. 2000), the regulation of their expression in trichomes remains obscure. Here we show that JA treatment of intact plants leads to higher transcript levels of *LeMTS1* mainly in trichomes (Fig. 7). In contrast, *WIPI-II* expression is induced by JA in the complementary tissues, indicating that there is a tissue-specific differential activation of JA-regulated genes. Basal expression of *WIPI-II* was very low in tomato trichomes, although constitutive expression of a *WIPI-II* in glandular trichomes was recently demonstrated in nightshade (*Solanum americanum*) (Liu et al. 2006). It is unknown whether trichome-expressed *WIPI-II* plays a role in the defense against herbivores. Induction of *WIPI-II* in non-glandular tissue (mesophyll/parenchyma) meets the expectation that it should be present in those cells that suffer from herbivory.

Previously, JA has been shown to increase trichome density on newly formed leaves of, for instance, Arabidopsis and tomato (Boughton et al. 2005; Traw and Bergelson 2003). Also, JA might induce trichome-based defenses directly. The production of acylsugars on the leaf surface (probably in trichomes) of *Datura wrightii* plants increased without affecting trichome density (Hare and Walling 2006). More clearly, nornicotin production on the *Nicotiana repanda* plant surface increased twofold within 6 h of JA treatment (Laue et al. 2000). Although it should be kept in mind that in the two above-mentioned studies, it was perhaps unjustly assumed that $CHCl_3$ or CH_2Cl_2 extraction releases only trichome contents, it indicates that trichome-based secondary metabolite biosynthesis can be inducible. Results from our study show, for the first time, a specific transcriptional regulation of a gene involved in secondary metabolite biosynthesis in trichomes. Isolation of trichomes revealed that linalool synthase expression in tomato trichomes was induced more than twofold by JA after 24 h.

In this paper, we report the identification of the first two monoterpene synthases from a plant of the solanaceae family. As it has recently been described that increased volatile terpene production in transgenic plants can benefit the indirect defense (Kappers et al. 2005; Schnee et al. 2006), it will be interesting to investigate the biological effect of *LeMTS1* overexpression in tomato. Furthermore, the role of LeMTS2-derived terpenes in roots and stems remains to be investigated. Finally, the specific induction of LeMTS1 in trichomes by JA can provide an excellent opportunity for identification of novel, JA-related promoter elements, and transcription factors.

Materials and methods

Isolation of *LeMTS1* and *LeMTS2* cDNAs

Tomato plants, *Lycopersicon esculentum* cv. Moneymaker (3–4 weeks old) were infested with 150 spider mites (*Tetranychus urticae*) as described by Kant et al. (2004). Leaves were collected and pooled after 2–5 days of infestation. RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA; cDNA was made using SuperscriptII RNAse H⁻ (Invitrogen). A *LeMTS* fragment was amplified from this cDNA using the primer 5′-GAT-GACATTTATGATGTTTATGGC-3′ in combination with an oligo dT(18) primer. The primer was designed based on putative tomato monoterpene synthases (www.tigr.org: TC168035, TC160168, BG131411, and cLED9K13) as such that it should not anneal to sesquiterpene synthase cDNAs. The 850 bp PCR product was cloned into pGEM-T easy (Promega, Madison, WI, USA) and sequenced using the ABI PRISM BigDye terminator kit (Applied Biosystems, Foster city, CA, USA). cDNA was synthesized from leaves of 3-week-old Moneymaker plants and from hypocotyls and roots of 11-week-old Moneymaker plants with a ZAP-cDNA synthesis kit, cloned into the Lambda-ACT vector (Elledge et al. 1991) and packaged with a ZAP-cDNA Gigapack II gold cloning kit (Stratagene, La Jolla, CA, USA) 200,000 plaques of each library were screenend with the 850 bp RT-PCR product as probe, radioactively labeled by the ALL-IN-ONE Random prime labeling method (Sigma, Saint Louis, MI, USA). Filters were hybridized at 55°C in BLOTTO hybridization buffer (Sambrook and Russell 2001) and washed three times for 10 min in 1 × SSC, 0.1% SDS at 55°C. DNA from positive plaques was converted to pAct2 plasmids and their inserts were sequenced. *LeMTS1* was obtained from the leaf cDNA library, *LeMTS2* from the hypocotyl and root cDNA library. Sequences can be found in Genbank as AY840091 and AY840092, respectively. The presence of plastid targeting signals was predicted using PREDOTAR (http://genoplante-info.infobiogen.fr/predotar/predotar.html; Small et al. 2004) and TargetP (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al. 2000).

Expression of recombinant proteins in *E. coli*, enzyme assays and product analysis

LeMTS1 and *LeMTS2* were subcloned into the pET32-a expression vector (Novagen, San Diego, CA, USA) after

removal of the plastid targeting signal sequences up to one amino acid upstream of the conserved arginine pair (Fig. 1) by generating 'truncated' *LeMTS1* and *LeMTS2* PCR products with *Pfu* DNA polymerase (Stratagene). Forward primers contained NcoI- and reverse primers XhoI-restriction sites. *LeMTS1* primers (5′–3′): GAT<u>CCATGG</u>ACA-CAAGGCGTTCAGGGAATTAC and GTA<u>CTCGAG</u>CAA AGTAATAAAATGAAGCCTACG, *LeMTS2* primers: GAT<u>CCATGG</u>GTATCCGACGTTCAGGAAATTAC and GTA<u>CTCGAG</u>AATAAAAGGTAA TAATTCCTTGTC. pET32-*LeMTS* constructs were transformed into *E. coli* BL21(DE3) which were selected on Luria-Bertani (LB) plates containing 100 mg/l ampicillin. A single colony was grown overnight at 37°C on a plate, and transferred to 100 ml of LB with ampicillin. Cultures were grown at 37°C for 30–60 min to A_{600} of 0.6–1.0 before addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside and grown at 20°C for an additional 8 h. Cells were harvested by centrifugation and resuspended in 5 ml monoterpene synthase assay buffer containing 50 mM HEPES pH 7.5, 10% glycerol, 5 mM DTT, 2 mM $MnCl_2$, 10 mM $MgCl_2$, proteinase inhibitor cocktail Complete (Roche, Basel, Switzerland), and 1 mM ascorbic acid. Cells were disrupted by addition of 1 mg/ml lysozym (Sigma), incubation on ice for 30 min and sonication. After centrifugation (10,000g) for 30 min at 4°C, 1 ml of the soluble enzyme fraction was assayed directly, or after purification of the His-tagged recombinant fusion protein on Ni-agarose beads (Novagen) for monoterpene, sesquiterpene or diterpene synthase activity using 10 μM of the substrates GPP, FPP or GGPP, respectively. The assay mix was incubated in a closed 20 ml vial at 30°C for 1 h under gentle shaking (150 rpm). Reaction products were sampled with a Solid Phase Micro Extraction fiber (SPME) for 10 min during which the vial was agitated and heated to 50°C. The SPME fiber was desorbed 1 min in an Optic injector port (ATAS GL Int., Zoeterwoude, The Netherlands) which was kept at 250°C. Compounds were separated on a DB-5 column (10 m × 180 μm, 0.18 μm film thickness; Hewlett Packard, Palo Alto CA, USA) in an 6,890 N gas chromatograph (Agilent, Amstelveen, The Netherlands) with a temperature program set to 40°C for 1.5 min, ramp to 250°C at 30°C/min and 250°C for an additional 2.5 min. Helium was used as carrier gas, the column flow was set to 3 ml/min for 2 min and to 1.5 ml/min thereafter. Mass spectra were generated with the ion source set to –70 V at 200°C and collected with a Time-of-Flight MS (Leco, Pegasus III, St. Joseph, MI, USA) at 1,671 V, with an acquisition rate of 20 scans/s. Terpene products were identified using authentic standards and comparison of ion specra and relative retention times (Adams 2001). For separation of terpene enantiomers, a Cyclosil-B column (30 m × 247 mm, 0.25 μm film thickness; Agilent) was used. The temperature program was isothermal at 115°C for 15 min, with a final

ramp to 240°C at 120°C/min and 240°C for 5 min. Column flow was 0.7 ml/min.

Plant treatments, plant headspace sampling, trichome isolation, and measurement of extracts

For determining tissue specific gene expression, a 13 weeks old, untreated tomato plant was dissected to obtain material of each of the described tissues. For separation of trichomes from petioles and stems, 4-week-old plants were used. Trichomes were collected on the bottom of a 50 ml tube after vortexing several $N_2(l)$-frozen petiole or stem segments, standing in upright position. The trichome fraction was used directly, whereas the remaining cleaned petiole or stem segments were thoroughly brushed to remove all remaining trichome material. Wounding-, spider mite-, and hormone-treatments (for both volatile sampling and gene expression analysis) were done using 3–4-week-old tomato plants. Wounding was inflicted by squeezing leaflets several times with a hemostat; a total of 150 spider mites were put on 3–4 leaflets per plant for 3 days; JA and salicylic acid were applied to plants by spraying 1 mM solutions made with tap water containing 0.05% SilwetL-77. Wounding- and hormone-treatments were done (cumulative) at 0 h and at 18 h and leaves were sampled at 24 h. Headspace sampling of plants was performed as described by Kant et al. (2004). Tenax sampling tubes were eluted with pentane : diethyl ether (4:1). The Eluted fractions were concentrated 20 times under a stream of N_2 and 1 μl was injected into the GC injector port. For root-, leaf-, trichome-, stem- or petiole-extracts, 0.5 g tissue was used. The amount of trichome material was adjusted to be equivalent to the trichomes present on 0.5 g of the original stem or petiole. Tissues were ground in $N_2(l)$ and transferred to glass 20 ml vials containing 2 ml saturated $CaCl_2$ (5 M) buffered in 100 mM sodium acetate (pH 4.5) which were capped immediately and kept at 5°C. Extracts were pre-incubated for 5 min at 60°C under agitation (500 rpm) and sampled for 10 min at 60°C on a 100 μM PDMS SPME fiber (Supelco, Zwijndrecht, The Netherlands). The SPME fiber was desorbed 1 min in the injector port of the GC/MS, which was kept at 250°C. GC/MS analysis was done as described earlier (Kant et al. 2004).

Gene expression analysis by RT-PCR and real-time Q-RT-PCR

For semi-quantitative RT-PCR, the tomato RUB1 conjugating enzyme (*RCE1*) was used as constitutively expressed control gene (www.tigr.org: TC153679). Initial PCR was performed with *RCE1* primers, product levels were com-

pared and individual cDNA samples were diluted accordingly, to ensure equal template concentrations. PCRs with primers of genes of interest were subsequently performed for non-saturating number of amplification cycles. *RCE1* primers were always included in each experiment. Used primers (5′–3′) for *RCE1*: forward (F) GATTCTCTC TCATCAATCAATTCG; reverse (R) GCATCCAAACTT-TACAGACTCTC, *WIPI-II*: F: GACAAGGTACTAGT AATCAATTATCC; R: CACATAACACACAACTTTGAT GCC, *PRP6*: F: TCAGTCCGACTAGGTTGTGG; R: TAG ATAAGTGCTTGATGTGCC, *LeMTS1*: F: GATGACAT TTATGATGTTTATGGC; R: GGCCATCTCGAGACTTG AGAGCGAATGCAACATTAG, *LeMTS2*: F: GATGA CATTTATGATGTTTATGGC; R: GGGTAATAATTC CTTGTCTTATTTC. Expression differences were validated by varying the amount of PCR cycles.

For real-time Q-RT-PCR, total RNA was isolated using Trizol (Invitrogen) and DNA was subsequently removed with DNAse (Ambion, Huntingdon, UK). cDNA was synthesized from 5 μg RNA using SuperscriptII (Invitrogen) in 20 μl reaction volume that was diluted to 50 μl prior to using it for PCR. PCRs were performed in the ABI 7500 Real-Time PCR System (Applied Biosystems) using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). About 20 μl PCR reactions contained 0.25 μM of each primer, 0.1 μl ROX reference dye, and 1 μl template. The cycling program was set to 2 min 50°C, 5 min 95°C, 40 cycles of 15 s at 95°C and 1 min 60°C, and a melting curve analysis. Primer pairs were tested for specificity and for linearity with a standard cDNA dilution curve. Primers used: *LeRCE1* QF: 5′-GATTCTCTCTCATCAATCAATTCG-3′ QR: 5′-GA-ACGTAAATGTGCCACCCATA-3′, *LeWIPI2* (K03291) QF: 5′-GACAAGGTACTAGTAATCAATTATCC-3′ QR: 5′-GGGCATATCCCGAACCCAAGA-3′, *LeMTS1* (AY8 40091) QF: 5′-TTTGGGGACATCTTCGGATGAA-3′ QR: 5′-CTACTCGAGTTACTTGAGAGCGAATG   CAAC-3′. Expression levels were normalized using *RCE1* mRNA levels.

Generation of transgenic tomato plants

The complete *LeMTS1* cDNA, including the putative signal peptide, was cloned into the EcoRI-digested and Klenow-blunted binary vector pGreen1K (Brandwagt et al. 2002), resulting in a CaMV35S:MTS1:nos cassette. The construct was transferred to *Agrobacterium tumefaciens* strain EHA105 carrying the pSoup helper plasmid (Hellens et al. 2000). Tomato plants cv. Moneymaker were transformed essentially as described by Vanroekel et al. (1993). Heterozygous and homozygous *LeMTS1* overexpressing plants were used to measure linalool production by sampling the headspace of intact plants (four sets of two plants for both *LeMTS1* overexpressors and control plants).

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