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ORIGINAL ARTICLE

Induction of a leaf specific geranylgeranyl pyrophosphate synthase and emission of (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene in tomato are dependent on both jasmonic acid and salicylic acid signaling pathways

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Abstract Two cDNAs encoding geranylgeranyl pyrophosphate (GGPP) synthases from tomato (*Lycopersicon esculentum*) have been cloned and functionally expressed in *Escherichia coli*. *LeGGPS1* was predominantly expressed in leaf tissue and *LeGGPS2* in ripening fruit and flower tissue. *LeGGPS1* expression was induced in leaves by spider mite (*Tetranychus urticae*)-feeding and mechanical wounding in wild type tomato but not in the jasmonic acid (JA)-response mutant *def-1* and the salicylic acid (SA)-deficient transgenic *NahG* line. Furthermore, *LeGGPS1* expression could be induced in leaves of wild type tomato plants by JA- or methyl salicylate (MeSA)-treatment. In contrast, expression of *LeGGPS2* was not induced in leaves by spider mite-feeding, wounding, JA- or MeSA-treatment. We show that emission of the GGPP-derived volatile terpenoid (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) correlates with expression of *LeGGPS1*. An exception was MeSA-treatment, which resulted in induction of *LeGGPS1* but not in emission of TMTT. We show that there is an additional layer of regulation, because geranyllinalool synthase, catalyzing the first dedicated step in TMTT biosynthesis, was induced by JA but not by MeSA.

Keywords Geranylgeranyl pyrophosphate · Jasmonic acid · *Lycopersicon* · Salicylic acid · Spider mites · Homoterpene

Abbreviations

GGPP	Geranylgeranyl pyrophosphate
JA	Jasmonic acid
SA	Salicylic acid
MeSA	Methyl salicylate
TMTT	(*E,E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene
FPP	Farnesyl pyrophosphate
GPP	Geranyl pyrophosphate
IPP	Isopentenyl pyrophosphate
GL	Geranyllinalool
DMNT	4,8-Dimethylnona-1,3,7-triene

Introduction

Geranylgeranyl pyrophosphate (GGPP) synthase belongs to a group of short-chain prenyltransferases that also include farnesyl pyrophosphate (FPP) synthase and geranyl pyrophosphate (GPP) synthase. These enzymes are involved in isoprenoid biosynthesis and share high sequence homology (Joly and Edwards 1993). An early step in isoprenoid biosynthesis, catalyzed by GPP synthase, is the condensation of two C5 molecules, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) forming GPP (C10). In plants two

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separate pathways exist for the synthesis of these universal C5 intermediates; the cytosolic mevalonate pathway and the plastidial methyl-D-erythritol 4-phosphate pathway. Addition of two IPP molecules to one DMAPP molecule and the addition of one IPP molecule to GPP is catalyzed by FPP synthase, resulting in FPP (C15). GGPP synthase catalyzes the formation of GGPP (C20) by condensation of DMAPP with three IPP molecules or by the condensation of GPP with two IPP molecules or FPP with one IPP molecule (Burke and Croteau 2002). GGPP is the precursor for many different products in plants like diterpenes, gibberellins, carotenoids, the isoprenoid side chain of chlorophyll and it is used for protein prenylation. Based on the genome sequence Arabidopsis has 12 putative GGPP synthases (Lange and Ghassemian 2003). Zhu et al. (1997) and Okada et al. (2000) have characterized five of them and showed that they differ in expression patterns and subcellular localization, making it likely that individual GGPP synthases have very specific functions and are regulated accordingly.

(*E,E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene (TMTT) is a diterpene-derived volatile produced by many plants in response to herbivory. The biosynthesis of TMTT has been proposed by Boland and Gäbler (1989). They suggest that TMTT is produced by oxidative degradation of geranyllinalool (GL). This synthesis parallels the biosynthesis of 4,8-dimethylnona-1,3,7-triene (DMNT), which is likely formed by oxidative degradation of the sesquiterpene (*E*)-nerolidol (Boland et al. 1998). The diterpene GL is supposedly formed by an uncharacterized GL synthase from the common diterpene precursor GGPP.

Herbivore-induced volatiles can benefit plants by attracting natural enemies of the attacking herbivore (Sabelis et al. 2001). Tomato plants infested with spider mites emit a blend of volatiles that makes them attractive to predatory mites (*Phytoseiulus persimilis*), a natural enemy of spider mites. Spider mite-infested tomato plants emit significantly more TMTT than uninfested tomato plants (Kant et al. 2004). The role of TMTT in the attraction of predatory mites has been investigated with lima bean (*Phaseolus lunatus*) as model system (De Boer et al. 2004). Predatory mites prefer the odor source of lima bean infested with spider mites above that of lima bean infested with beet armyworm (*Spodoptera exigua*). Spider mite-infested lima bean emits significantly more TMTT than beet armyworm-infested lima bean. When TMTT is added to the odor of beet armyworm-infested plants, predatory mites prefer this odor above that of spider mite-infested plants. This suggests that TMTT can influence the foraging behavior of predatory mites.

Emission of TMTT in excised lima bean leaves can be induced by early intermediates of the jasmonic acid (JA) biosynthetic pathway, linolenic acid and 12-oxo-phytodienoic acid (Koch et al. 1999). There is evidence that, in tomato, emission of TMTT is dependent on JA. The tomato mutant *def-1*, which is deficient in induced JA-accumulation after wounding or herbivory (Li et al. 2002), does not emit TMTT upon spider mite-infestation. However, emission of TMTT can be restored by pre-treating these plants with JA (Ament et al. 2004). These results indicate that biosynthesis of TMTT is regulated by oxylipins.

Induced production of terpenes is often correlated to induced terpene synthase activity. Terpene synthases generate specific products from common terpene precursors. Regulation at the level of terpene synthases allows production of a highly specific terpene blend. However, there are also some reports on the regulation of genes encoding enzymes that act upstream of terpene synthases, resulting in increased precursor pools. In cytosolic IPP synthesis, 3-hydroxy-3-methylglutaryl-coenzyme A reductase is upregulated in response to pathogens or pathogen-derived elicitors in solanaceous plants like tomato (Park et al. 1992), Korean red pepper (*Capsicum annuum*) (Ha et al. 2003) and potato (*Solanum tuberosum*) (Choi et al. 1992), but also by insect herbivores in potato (Korth et al. 1997) and *Nicotiana attenuata* (Hui et al. 2003). Furthermore, Kant et al. (2004) have shown that transcription of deoxy-xylulose-5-phosphate synthase, involved in plastidial IPP synthesis, is upregulated in tomato upon spider mite-infestation. Downstream, at the level of prenyltransferases that precede the action of terpene synthases, little is known about regulation of gene expression. FPP synthase, required for sesquiterpene biosynthesis, is upregulated by pathogens in Korean red pepper (Ha et al. 2003) and cotton (*Gossypium hirsutum*) (Liu et al. 1999) and by insect herbivores in maize (*Zea mays*) (Farag et al. 2005). GGPP synthase is induced in taxus cell suspensions by methyl jasmonate (Hefner et al. 1998) and by spider mite-herbivory in tomato (Kant et al. 2004).

To investigate the regulation of TMTT production in tomato we cloned two GGPP synthases from tomato (*LeGGPS1*, DQ267902 and *LeGGPS2*, DQ267903), of which *LeGGPS1* is proposed to be involved in precursor biosynthesis for TMTT. We also determined the role of the phytohormones JA and salicylic acid (SA) in regulating *LeGGPS1* expression and GL synthase activity, leading to TMTT emission.

Materials and methods

Chemicals

TMTT was synthesized by Prof. H. Hiemstra (Department of synthetic organic chemistry, University of Amsterdam, The Netherlands) as described by Dodd and Oehlschlager (1992). Purity exceeded 98% as was checked by NMR and GC-MS. Geranyllinalool, benzyl acetate and methyl salicylate (MeSA) were obtained from Fluka (Buchs, Switzerland) and JA from Duchefa Biochemicals (Haarlem, the Netherlands).

Plant material and arthropod rearing

Tomato seedlings [*Lycopersicon esculentum* Mill cv. Castlemart, *def-1* (Howe and Ryan 1999), cv. Moneymaker (obtained from the Glasshouse Crop Research Institute (GCRI), now Horticultural Research Institute (HRI), UK) and *NahG* (Brading et al. 2000)] were grown as described by Kant et al. (2004). The two-spotted spider mite (*Tetranychus urticae* Koch) was originally obtained in 1993 from tomato plants in a greenhouse (Houten, The Netherlands; Gotoh et al. 1993) and was maintained on the cultivar Moneymaker ever since.

Plant treatments

Plants used in all experiments, except for tissue specific expression, were 18–21 days old and had four fully expanded leaves and two emerging leaves. Three days prior to the start of the experiments, plants were transferred to a climate chamber. For tissue specific expression 14-week-old plants were used that were grown in the green house. Adult female spider mites were gently placed on the adaxial surface of the fully expanded terminal leaflets using a soft-bristle paintbrush. Per leaflet 15 mites were introduced, 3 leaflets on each plant. Mechanical damage was inflicted on three leaflets per plant with three incisions of an artery clamp per leaflet. Plants were treated with JA by misting them with 3 ml of a 0.25 mM solution. Plants were left to recover for 24 h before the start of the experiment. For the MeSA treatments a single plant was enclosed in an airtight glass desiccator of 22 l. On a cotton swab 16.7 mg MeSA was applied, which upon evaporation resulted in a vapor concentration of 5 μM. After 24 h plants were transferred to clean desiccators for volatile analysis or harvested for RNA isolation.

Volatile analysis

Single plants were placed in a glass desiccator of 22 l that was ventilated with carbon filtered air at 200 ml/min. The headspace (the air around the plant) was sampled during 24 h for 2 consecutive days by trapping the outgoing air on 300 mg Tenax TA (Alltech, Deerfield, IL, USA) in a 5-mm wide glass tube. The complete volatile collection set-up was made from glass and Teflon and no grease was used. The headspace of spider mite-infested plants was collected on the fourth day of spider mite-infestation. Simultaneously, clean plants were enclosed in similar desiccators as control treatment. Volatiles of mechanically wounded plants were sampled for 24 h directly after damaging the plants. After 24 h of treatment with JA or MeSA, plants were placed in clean desiccators and volatiles were sampled in the subsequent 2 days in 24-h intervals. The Tenax tubes were eluted with 2 ml pentane:diethylether (4:1, v/v) with 1.8 μg of benzyl acetate as internal standard. One microliter was analyzed using gas chromatograph mass-spectrometry as described by Ament et al. (2004). Compounds were identified and quantified on the basis of the internal standard and synthetic external standards of known concentrations. All experiments were performed twice.

RT-PCR analysis

For determining tissue specific expression of *LeGGPS1* and *LeGGPS2*, a mature plant was dissected and tissues were frozen in liquid nitrogen and stored at −80°C. To determine induction of *LeGGPS1* or *LeGGPS2*, leaves were taken from spider mite-infested, wounded, JA-, MeSA- and control plants. Experiments with *NahG*, *def-1* and the corresponding wild type tomato plants, infested with spider mites and control leaves, were performed in a climate chamber. Tissues were harvested as described above. Total RNA was isolated with a Phenol-LiCl based method as described by Verdonk et al. (2003). RT-PCR determination of transcript levels was done as described by Ament et al. (2004). For each cDNA we determined the number of cycles for which the PCR amplification was not saturated. PCR products were visualized by staining with ethidium bromide after separation on agarose gels. Quantification of transcript levels of *LeGGPS1* and RUB1 conjugating enzyme (*RCE1*) was performed as described by Ament et al. (2004). PCR amplification of *RCE1* fragments (0.723 kb) comprised 20 cycles with forward primer, 5′-GATTCTCTCTCAT CAATCAATTCG-3′ and reverse primer, 5′-GCATC CAAACTTTACAGACTCTC-3′; of *PRP6* fragments (0.427 kb) 23 cycles with forward primer 5′-TCAGTC CGACTAGGTTGTGG-3′ and reverse primer 5′-TA GATAAGTGCTTGATGTGCC-3′ of *WIPI-2* fragments (0.601 kb) 14 cycles with forward primer, 5′-GA

CAAGGTACTAGTAATCAAT-3′ and reverse primer, 5′-CACATAACACACAACTTTGATGCC-3′; of *LeGGPS2* fragments (0.378 kb) 25 cycles with forward primer 5′-CTTTGGATGAGGCTATAATGG-3′ and reverse primer 5′-ATTCGGCGACAGCAACGAGG-3′; and of *LeGGPS1* fragments (0.327 kb) 27 cycles with forward primer 5′-GCAATCAAGGTAAACAAAGCAC-3′ and reverse primer 5′-CAAAGATAAAAGTGCATCCCCTG-3′. All PCR amplifications were performed with each cycle: 94°C for 45 s, 55°C for 45 s and 72°C for 75 s and a final extension of 5 min at 72°C. RT-PCR products were sequenced to confirm specific amplification.

Phylogenetic analysis

The sequences of *LeGGPS1*, *LeGGPS2* and several other prenyltransferases present in the NCBI GenBank database were aligned using the CLUSTALW algorithm in MacVector (Oxford Molecular Ltd., Oxford, UK). This alignment was used to construct a mid point rooted tree with Neighbor joining method. The distance was Poisson-corrected and gaps were distributed proportionally. Bootstrap analysis was performed with 100 replications.

Genetic complementation of E. coli with *LeGGPS1* and *LeGGPS2*

The plasmid pACCAR25Δ*crtE* (Sandmann et al. 1993) contains the gene cluster from *Erwinia uredovora*, with *crtB*, *crtI*, *crtX*, *crtY* and *crtZ* encoding carotenoid biosynthetic enzymes, with the exception of *crtE* (encoding GGPP synthase). Full-length cDNAs of *LeGGPS1* and *LeGGPS2* were cloned into pBluescript KS+ to produce a LacZ fusion protein. As a positive control, pBluescript containing human (*Homo sapiens*) GGPP synthase as a fusion with *LacZ* was used, and as a negative control pBluescript without an insert. pBluescript plasmid containing human GGPP synthase and pACCAR25Δ*crtE* were kindly provided by Dr. M. Kawamukai, Shimane University, Shimane, Japan. DH10β *Escherichia coli* cells that had previously been transformed with the pACCAR25Δ*crtE* plasmid were transformed with pBluescript containing *LeGGPS1*, *LeGGPS2*, human *GGPS* or no insert. Transformants were plated on LB agar medium containing 50 μg/ml ampicillin, 25 μg/ml chloramphenicol and 1 mM IPTG, and incubated at 30°C for 24 h. Subsequently, independent colonies were transferred to liquid LB medium containing 50 μg/ml ampicillin, 25 μg/ml chloramphenicol and 1 mM IPTG and grown at 30°C for 24 h. Cells from 2 ml of these cultures were harvested by centrifugation. These cells were extracted with 1 ml of 90% acetone, and the absorption at 450 nm was determined as a measure for carotenoid production.

Geranyllinalool synthase assay

Frozen leaf tissue (1.5 g) was ground in liquid nitrogen, and homogenized further with a Polytron (Kinematica AG, Luzern, Switzerland) in 4 ml extraction buffer containing 100 mM hepes (pH 8.0), 10 mM ascorbic acid, 50 mM sodium meta-bisulphite, 20% glycerol, 10 mM $MgCl_2$, 5 mM DTT, proteinase inhibitor cocktail complete (Roche, Mannheim, Germany) and 0.4 g PVPP. Extracts were incubated with 0.8 g Amberlite XAD-4 for 5 min, filtered through Miracloth and centrifuged for 20 min at 20,000 *g*. Three milliliter supernatant was rebuffered, using Econo-Pac 10DG columns (Bio Rad), to assay buffer containing 50 mM hepes (pH 8.0), 1 mM ascorbic acid, 10% glycerol, 10 mM $MgCl_2$, 1 mM DTT and proteinase inhibitor cocktail complete. Assays were performed in 10 ml glass vials with 1 ml desalted extract and were incubated for 1 h at 30°C with 6 μM GGPP (Sigma). Assay products were sampled on a 100 μM PDMS solid phase micro extraction (SPME) fiber (Supelco, Zwijndrecht, The Netherlands) for 15 min at 60°C. The SPME fiber was desorbed for 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) in an 6890N gas chromatograph (Agilent, Amstelveen, The Netherlands) with a temperature program set to 40°C for 1.5 min, ramp to 280°C at 30°C min^{-1} 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. The product GL was identified using the mass spectrum and retention time of the authentic standard and quantified using a dilution series of the authentic standard in the same quantity-range in desalted extract.

Results

Identification of two GGPP synthases from tomato

We had previously identified two tomato cDNAs encoding putative GGPP synthases (http://www.tigr.org TC168578 and TC167026), which were differentially

regulated by spider mite-herbivory (Kant et al. 2004). The full-length cDNAs, *LeGGPS2* and *LeGGPS1*, were generated by 5′ RACE PCR using reverse transcribed cDNA from tomato flower tissue and spider mite-infested tomato leaf tissue, respectively. The open reading frames of *LeGGPS1* and *LeGGPS2* were predicted to encode proteins of 365 and 363 amino acids with a mass of 39.9 and 39.0 kDa. TargetP and ChloroP (Emanuelsson et al. 2000) software predicted a plastid localization peptide for both *LeGGPS1* and *LeGGPS2*. The deduced mature protein sequences of *LeGGPS1* and *LeGGPS2* share 68% identity. Both proteins contain the seven highly conserved domains present in prenyltransferases (Koike-Takeshita et al. 1995). The three aspartic acid residues and the two arginine residues in domain II as well as the FQXXD-DXLD motif in domain VI, involved in substrate binding (Kellogg and Poulter et al. 1997), were conserved in both tomato GGPP synthase proteins (Supplemental Fig. 1). Figure 1 shows a phylogenetic tree based on the deduced amino acid sequences of *LeGGPS1*, *LeGGPS2* and several other prenyltransferases. This analysis indicates that these two tomato GGPP synthases cluster with other plant GGPP synthases and form a branch different from the FPP synthases and the GPP synthases. The large subunits of the heterodimeric GPP synthases from snapdragon and mint also cluster in the group of GGPP synthases, whereas the small subunits cluster as an out-group. Of these large subunits it is known that they exhibit GGPP synthase activity, when expressed in *E. coli* without the small subunit (Tholl et al. 2004).

Functional expression of *LeGGPS1* and *LeGGPS2 in E. coli*

To confirm that *LeGGPS1* and *LeGGPS2* are functional GGPP synthases, we expressed full-length cDNAs in *E. coli* harboring the gene cluster from *Erwinia uredovora* containing all genes for carotenoid biosynthesis (*crtB*, *crtI*, *crtX*, *crtY*, *crtZ*), except GGPP synthase (*crtE*). If the product of the introduced gene has GGPP synthase activity, these cells will produce carotenoids and become orange. *LeGGPS1* and *LeGGPS2* could indeed complement *E. coli* cells missing *crtE*. Human GGPP synthase was used as positive control. The empty vector control did not show carotenoid production, which was measured spectrophotometrically in cell extracts at 450 nm (Fig. 2).

LeGGPS1 and *LeGGPS2* are differentially expressed in various plant tissues

The TIGR tomato database contains two ESTs corresponding to *LeGGPS1*, both present in the library made from tomato leaves infected with the bacterial pathogen *Pseudomonas syringae*. Three ESTs from *LeGGPS2* are present in the TIGR database, all in the library made from fruit at the breaker stage. This suggests tissue-specific expression of these two genes. To determine this tissue-specific expression in more detail, we dissected a mature tomato plant and isolated RNA from different tissues for RT-PCR analysis with gene specific primers. This showed that *LeGGPS1* transcripts are predominantly present in leaf tissue,

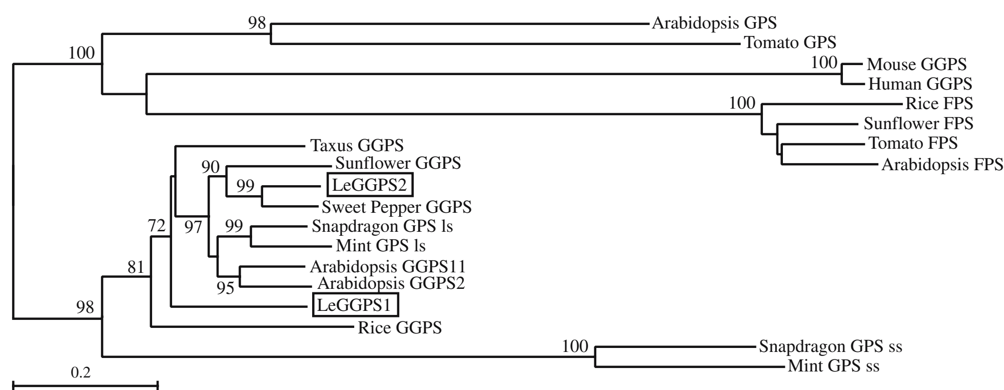

Fig. 1 *LeGGPS1* and *LeGGPS2* cluster with other plants geranylgeranyl pyrophosphate (GGPP) synthases. A midpoint rooted phylogenetic tree of Arabidopsis GPS (AB104727), tomato GPS (DQ286930), mouse GGPS (AB106044), Human GGPS (BC067768), rice FPS (NM192229), sunflower FPS (AF019892), tomato FPS (AF044747), Arabidopsis FPS (L46367), Taxus GGPS (AY566309), sunflower GGPS (AF020041), *LeGGPS2* (DQ267903), sweet pepper GGPS (X80267), snapdragon GPS ls (AY534687), mint GPS ls (AJ249453), Arabidopsis GGPS11 (L25813), Arabidopsis GGPS2 (AC006135), *LeGGPS1* (DQ267902), rice GGPS (NM188788), snapdragon GPS ss (AY534686), mint GPS ss (AF182827) is shown. Bootstrap values are indicated at the branch points. The tomato GGPP synthases are *boxed*. Small subunits of GPS are indicated with *ss* and large subunits with *ls*

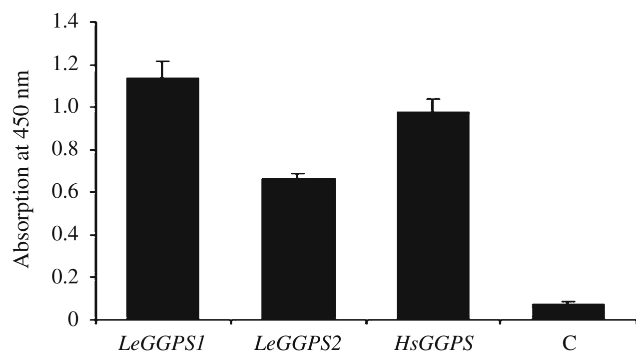

Fig. 2 *LeGGPS1* and *LeGGPS2* encode functional GGPP synthases. Absorption at 450 nm of extracts from cells expressing *LeGGPS1*, *LeGGPS2*, human GGPS (*HsGGPS*) or empty vector (*C*) is depicted, which is indicative for carotenoid production. Mean and standard deviation are shown of three measurements on independent transformants

whereas *LeGGPS2* transcripts are most abundant in fruits and flower organs (Fig. 3).

Expression of *LeGGPS1* and emission of TMTT are induced by spider mites and mechanical wounding

TMTT, the most abundant spider mite-induced tomato volatile, is probably synthesized from GGPP via GL. When spider mites forage, leaves are continuously damaged and this wounding leads to a wound response. To investigate whether emission of TMTT is specific for spider mite-feeding or a result of the wounding, we sampled the headspace of spider mite infested tomato plants and of mechanically wounded tomato plants. The mechanical wounding of plants was inflicted with an artery clamp. Even though this type of damage is in not comparable to spider mite-damage since the plants were wounded only at one time point, the area of the leaf that is damaged by these treatments is comparable. Volatiles were collected in the subsequent 24 h. Both spider mite-feeding and mechanical wounding resulted in the emission of comparable amounts of TMTT, indicating that wounding is sufficient to induce TMTT emission (Fig. 4a). This situation is apparently different from that in lima beans, where emission of TMTT by mechanically wounded leaves was significantly less than by caterpillar-wounded leaves (Mithöfer et al. 2005).

Since there is no substantial storage of TMTT in leaves of tomato plants (Ament et al. 2004), TMTT must be synthesized de novo, as Farag and Pare (2002) have demonstrated by labeling experiments with $^{13}CO_2$. We hypothesized that the pool of GGPP necessary for TMTT biosynthesis must be renewed and thus investigated expression of *LeGGPS1* and *LeGGPS2* after spider mite-feeding and mechanical wounding. Expression of *LeGGPS1* was spider mite- and wound-induced, correlating with induced emission of TMTT (Fig. 4). *LeGGPS2* was not induced by spider mite-feeding or by mechanical wounding (Fig. 4b). Spider

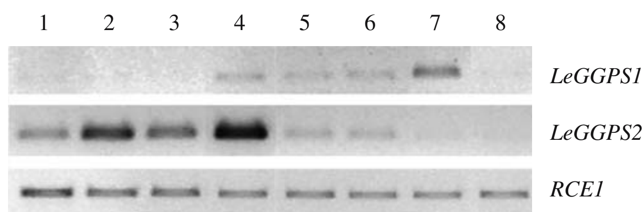

Fig. 3 *LeGGPS1* and *LeGGPS2* are differently expressed in various tissues. RT-PCR analysis for *LeGGPS1* (27 cycles), *LeGGPS2* (25 cycles) and *RCE1* (20 cycles) on RNA isolated from: (1) Green fruit, (2) Orange fruit, (3) Red fruit, (4) Carpel plus stamen plus ovary, (5) Sepal, (6) Stem, (7) Leaf and (8) Root. Rub1 conjugating enzyme (*RCE1*) is shown to indicate that equal amounts of template were used in the PCR reaction. A representative set of data from two independent experiments is shown

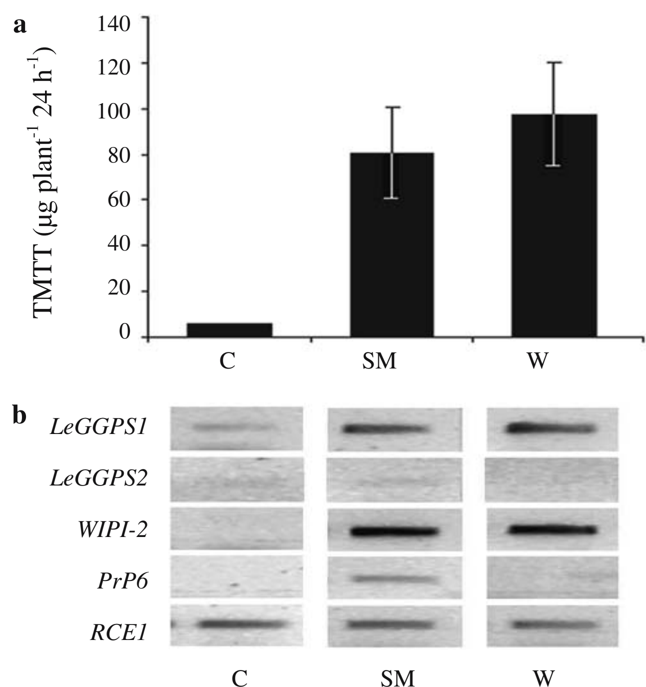

Fig. 4 *LeGGPS1* expression and TMTT emission are induced by spider mite-feeding and mechanical wounding. **a** *Bar graph* depicting the emission of TMTT in μg per plant in 24 h. Mean, minimum and maximum values are shown of measurements from two independent experiments. **b** RT-PCR analysis for *LeGGPS1* (27 cycles), *LeGGPS2* (25 cycles), *WIPI-2* (14 cycles), *PRP6* (23 cycles) and *RCE1* (20 cycles) on RNA isolated from leaves of control (*C*), spider mite-infested (*SM*) and mechanically wounded (*W*) plants. Rub1 conjugating enzyme (*RCE1*) is shown to indicate that equal amounts of template were used in the PCR reaction. Representative data of two independent experiments are shown

mite-feeding induced both the wound-induced proteinase inhibitor gene *WIPI-2*, a marker gene for a JA-response (Graham et al. 1985), and the gene encoding the pathogenesis related protein *PRP6* (also named *PR1b1*), a marker for the SA-dependent response (Tornero et al. 1997). Wounding alone did not induce *PRP6* expression.

Spider mite-induced emission of TMTT is both JA- and SA-dependent

To investigate whether emission of TMTT is dependent on JA or SA, we made use of the mutant *def-1* that is disturbed in the JA-response and the transgenic *NahG* line that overexpresses the *Pseudomonas putida* salicylate hydroxylase gene (*nahG*), resulting in inactivation of SA by converting it into catechol (Brading et al. 2000). *Def-1* plants have previously been shown to emit significantly lower amounts of TMTT than wild type plants after spider mite-attack (Ament et al. 2004). Here we show that SA is essential for emission of TMTT, since *NahG* plants emitted 50-fold less TMTT than wild type plants when infested with spider mites (Fig. 5).

LeGGPS1 is not a typical JA- or SA-responsive gene

Because TMTT production was abolished in the *NahG* line, we investigated whether this disruption of the SA-signaling pathway also abolished *LeGGPS1* expression. Indeed, *LeGGPS1* was not induced in *NahG* plants upon spider mite-feeding, while expression of the JA-responsive *WIPI-2* gene was much higher than in the wild type (Fig. 6a). Induction of *LeGGPS1* was also lower in *def-1* plants (Fig. 6b). In these *def-1* plants *WIPI-2* was not induced, but the induction of the SA-responsive *PRP6* gene was higher than in wild type plants (Fig. 6b). This cross talk between the networks of JA- and SA-responsive genes has been well documented (Spoel et al. 2003). However, *LeGGPS1* does not behave like a typical SA- or JA-responsive gene. In the absence of an SA-response, as in *NahG*, *LeGGPS1* was not induced although the induction of the JA-responsive gene *WIPI-2* was enhanced (Fig. 6a). Vice versa, in the absence of a JA-response in *def-1*, *LeGGPS1* was not stronger induced than in the wild type tomato, which is observed for the SA-responsive gene *PRP6* (Fig. 6b).

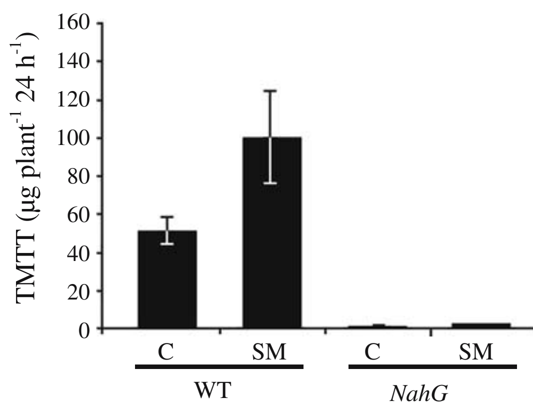

Fig. 5 NahG tomato plants do not emit TMTT. The *bar graph* depicts the emission of TMTT in µg per plant in 24 h by wild type (*WT*) and *NahG* plants infested with spider mites (*SM*) and control plants (*C*). Mean, minimum and maximum values are shown of measurements from two independent experiments

LeGGPS1 is induced by both JA and MeSA treatment but TMTT and GL are only emitted after JA treatment

To address the question whether JA or SA could induce *LeGGPS1* expression, we treated wild type tomato plants with these hormones. When wild type plants were treated with JA, *LeGGPS1* was induced (Fig. 6c). This JA treatment led to rapid induction of *WIPI-2* gene expression, while *PRP6* expression was not induced. When plants are treated with MeSA, this will be converted in the plant to SA by demethylation (Shulaev et al. 1997). This treatment led to activation of SA-responsive genes as could be seen from the induction of *PRP6*. *LeGGPS1* was also induced by this MeSA-treatment, whereas transcript levels of *LeGGPS2* and *WIPI-2* were not affected.

To investigate whether these hormone-treatments also induced emission of TMTT, the headspaces of these plants were sampled. JA-treated plants emitted 14-fold more TMTT than the control plants 2 days after the treatment (Fig. 7a). JA-treated plants emitted approximately 0.5 mg TMTT in 24 h, more than eight times the emission after spider mite-feeding or wounding. However, treatment of tomato plants with MeSA induced *LeGGPS1*, but did not lead to an increase in emission of TMTT (Fig. 7a). This lack of correlation suggests JA-dependent regulation of TMTT biosynthesis downstream of GGPP.

The first committed step in the biosynthesis of TMTT is the formation of GL from GGPP by GL synthase. Interestingly, the induction of GL emission coincided with the induction of TMTT emission (Fig. 7b). Two days after the treatments, about 1 µg GL was emitted in 24 h by MeSA-treated and control plants, whereas more than 20 µg GL was emitted by JA-treated plants. This suggests that GL synthase was activated by JA but not by SA.

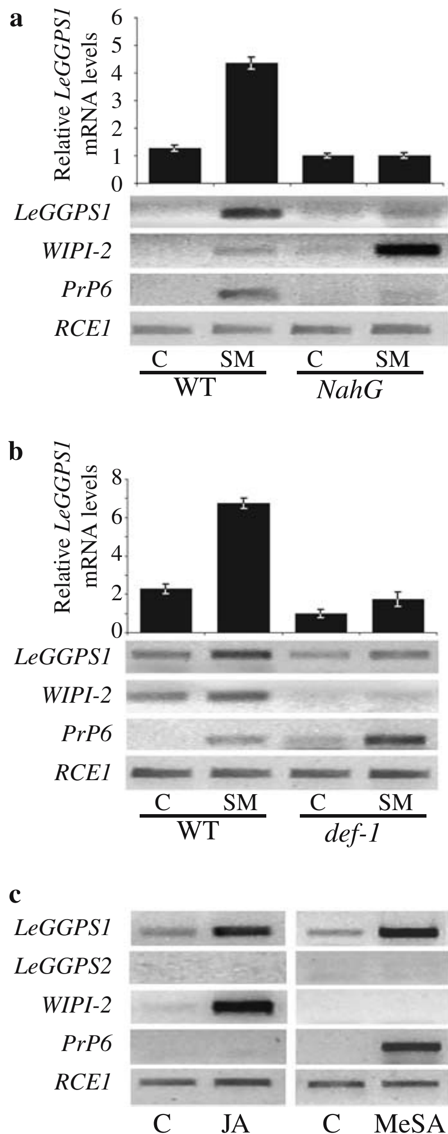

Fig. 6 *LeGGPS1* expression is dependent on JA and SA and is induced by JA-and MeSA-treatment. RT-PCR analysis for *LeGGPS1* (27 cycles), *LeGGPS2* (25 cycles), *WIPI-2* (14 cycles), *PRP6* (23 cycles) and *RCE1* (20 cycles) on RNA isolated from leaves of **a** *NahG* plants, **b** *def-1* plants and the corresponding wild type (*WT*) tomatoes, infested with spider mites (*SM*) and uninfested (*C*). The *bar graphs* represent quantification of blotted *LeGGPS1* RT-PCR products that were normalized for *RCE1* (means, minimal and maximal values are shown from two independent experiments). **c** RT-PCR analysis on RNA isolated from leaves of JA-, MeSA- and control (*C*)-treated plants. Rub1 conjugating enzyme (*RCE1*) is shown to indicate that equal amounts of template were used in the PCR reaction. A representative set of data of two independent experiments is shown

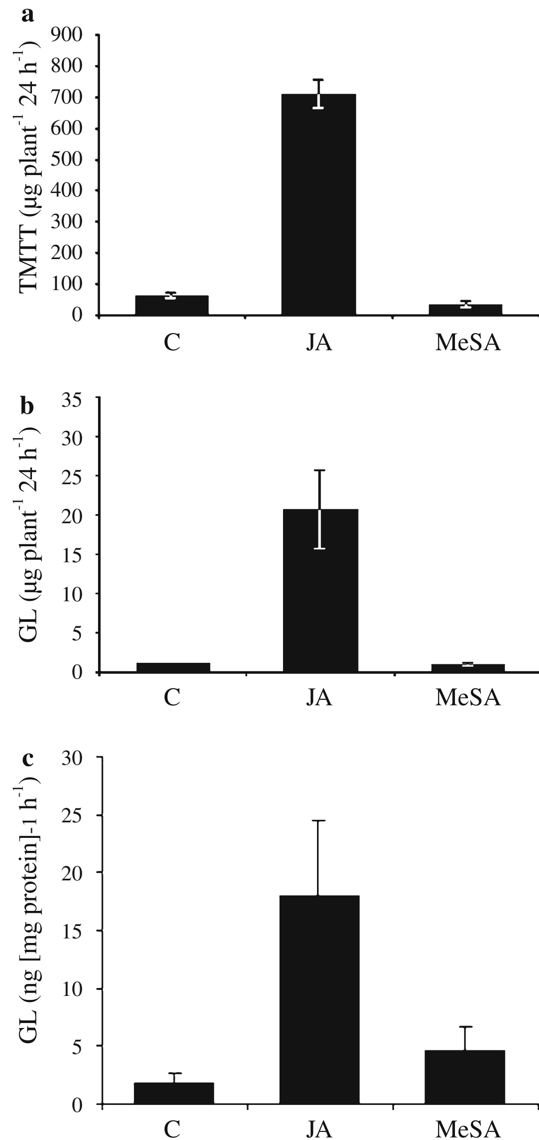

Fig. 7 Emission of TMTT and GL, and activity of GL synthase is induced by JA- but not by MeSA-treatment. *Bar graphs* depicting the emission of **a** TMTT and **b** GL in µg per plant in 24 h by JA-, MeSA- and control (*C*)-treated plants 2 days after treatment are shown. Mean, minimum and maximum values are shown of measurements from two independent experiments. **c** A *bar graph* depicting the production of GL in ng per h per mg protein. Mean and standard error are shown of measurements from four independent experiments

GL synthase activity in tomato leaves is induced by JA but not by SA

To test the hypothesis that GL synthase is induced by JA and not by SA, protein extracts from leaves of plants treated with either JA or MeSA were assayed for GL synthase activity. GL synthase activity was higher in JA-treated leaves compared to control-treated, whereas GL synthase activity in MeSA-treated leaves did not differ significantly from control-treated leaves (Fig. 7c). This indicates that GL synthase activity is induced by JA and not SA.

Discussion

Two GGPP synthase genes from tomato, *LeGGPS1* and *LeGGPS2*, have been cloned and expressed in *E. coli*. Functional expression of *LeGGPS1* and *LeGGPS2* was confirmed by complementation of the gene cluster for carotenoid biosyntheses of *Erwinia uredovora* lacking GGPP synthase (Fig. 2). This system has been used before to demonstrate the function of putative GGPP synthases from Arabidopsis (Zhu et al. 1997), sunflower (*Helianthus annuus*) (Oh et al. 2000) makandi (*Coleus forskohlii*) (Engprasert et al. 2004), human and mouse (*Mus musculus*) (Kainou et al. 1999).

While GGPP synthase, FPP synthase and most GPP synthases are functional as homodimers (Ogura and Koyama 1998), the GPP synthases from mint and snapdragon are functional as heterodimers (Burke et al. 1999; Tholl et al. 2004). In the phylogenetic tree (Fig. 1), the large subunits of these GPP synthases from mint and snapdragon cluster together with plant GGPP synthases. These large subunits exhibit GGPP synthase activity in the absence of the small subunit (Tholl et al. 2004). We therefore cannot exclude that *LeGGPS1* and *LeGGPS2* encode large subunits of tomato GPP synthases. However the tomato GPP synthase is highly similar to the single copy GPP synthase of Arabidopsis (accession number Y17376), which is functional as a homodimer (Bouvier et al. 2000). Moreover, in the TIGR tomato EST database, consisting of 160,000 ESTs, there are no ESTs with sequence similarity to the small subunit of GPP synthase of mint or snapdragon. We therefore reason that the GPP synthase of tomato is functional as a homodimer.

RT-PCR analysis showed that *LeGGPS1* and *LeGGPS2* were expressed in different tissues (Fig. 3). *LeGGPS1* was mostly expressed in leaf tissue while expression of *LeGGPS2* was highest in ripening fruit and flower organs (Fig. 3). During the first 5 days of spider mite infestation, a 4-week-old tomato plant emits around 900 μg volatiles of which approximately 75% is TMTT (Kant et al. 2004). Since *LeGGPS1* was mostly expressed in leaf tissue, we expect a leaf specific fate for the GGPP formed. Expression of *LeGGPS1* was induced by wounding and herbivory, coinciding with emission of TMTT (Fig. 4). We therefore hypothesize that *LeGGPS1* produces substrate for TMTT biosynthesis. It is possible that *LeGGPS2* provides GGPP for the production of the carotenoids in fruits, which increases from 2.2 μg/g FW in green fruit to 189 μg/g FW in red fruit (Fraser et al. 1994). The TIGR tomato EST database contains several ESTs encoding other putative GGPP synthases. It remains to be investigated whether they encode true GGPP synthases, where they are expressed and what regulates their expression.

Mechanical wounding and spider mite-herbivory induced expression of *LeGGPS1* in leaves. Both treatments resulted in a JA-response as could be seen from the induction of *WIPI-2* (Fig. 4b). Induction of *LeGGPS1* by spider mite-herbivory confirms the microarray results from Kant et al. (2004). Treatment of intact plants with JA induced the expression of *LeGGPS1* (Fig. 6c). This, in combination with reduced expression levels of *LeGGPS1* in the JA-deficient mutant *def-1* (Fig. 6b), implies JA-dependent regulation of *LeGGPS1*. Despite the inability of *def-1* to accumulate JA, *LeGGPS1* was at most slightly induced in response to spider mite-herbivory, compared to the wild type (Fig. 6b). Microarray experiments also did not show a significant induction of *LeGGPS1* in *def-1* plants by spider mites after 1 day (Ament et al. 2004). Due to the absence of induced JA-accumulation in *def-1*, the SA-responsive *PRP6* gene was stronger induced in *def-1* than in the wild type by spider mite-herbivory (Fig. 6b). To test whether this SA-response could account for the induction of *LeGGPS1* in *def-1*, we determined the expression of *LeGGPS1* after MeSA treatment of intact wild type plants. *LeGGPS1* was induced by this treatment, showing that SA can also induce *LeGGPS1* (Fig. 6c). Moreover, in the absence of SA, in the transgenic *NahG*, no induction of *LeGGPS1* occurred after spider mite-feeding (Fig. 6a), despite the induction of the JA-responsive *WIPI-2* gene. We therefore conclude that JA and SA can both induce *LeGGPS1* and that basic levels of SA are essential for the induction of *LeGGPS1*. Other examples of genes that are induced by JA- and SA-treatment have been described for sorghum (Salzman et al. 2005). Induction of several genes from Arabidopsis is also dependent on both JA- and SA-signaling pathways (Glazebrook et al. 2003). However, from these and other expression data (http://www.genevestigator.ethz.ch), no functional homolog of *LeGGPS1* could be pinpointed in Arabidopsis.

We have previously shown that emission of TMTT upon spider mite-herbivory was strongly reduced in the JA-deficient mutant *def-1* and that emission could be restored by pre-treating *def-1* with JA (Ament et al. 2004). When wild type plants were treated with JA they started to emit massive amounts of TMTT (Fig. 7a). Therefore, JA is necessary and sufficient to induce emission of TMTT in tomato. In lima bean leaves JA-treatment did not induce TMTT emission, though treatment with 12-oxo-phytodienoic acid (OPDA) and linolenic acid, both precursors of JA, did induce TMTT emission (Koch et al. 1999). Thus

regulation of TMTT emission is different for tomato and lima bean. *NahG* tomato plants did not emit TMTT after spider mite-herbivory, whereas wild type plants did (Fig. 5). However, treatment of wild type plants with MeSA did not result in induced emission of TMTT (Fig. 7a). Therefore we conclude that SA is not sufficient to induce the emission of TMTT but that a basic level of SA is required.

It must be noted that the corresponding wild type control plants for *NahG* and *def-1*, the cultivar Moneymaker and Castlemart, respectively, showed different transcript levels of *LeGGPS1*. This was also the case for *WIPI-2* (Fig. 6a, b), indicating that the JA-responses in these cultivars were different. The emission of TMTT by Moneymaker control plants (Fig. 5) was also much higher than by control Castlemart plants (Fig. 4). However, in both cultivars the emission of TMTT and expression of *LeGGPS1* were induced by spider mite-feeding.

Since MeSA- as well as JA-treatment resulted in induction of *LeGGPS1* whereas TMTT was only emitted after JA-treatment, there must be JA-dependent regulation of TMTT synthesis downstream of GGPP synthase. The first committed step in TMTT biosynthesis is the conversion of GGPP to GL, catalyzed by GL synthase. MeSA-treated plants emitted no GL whereas JA-treated plants did (Fig. 7b). Therefore we conclude that GL synthase must be JA-regulated. This conclusion is supported by the measurement of GL synthase activity in leaf extracts of JA-, SA- and control-treated plants: GL synthase activity was only induced after JA treatment (Fig. 7c).

TMTT biosynthesis parallels DMNT biosynthesis where FPP is converted to DMNT via nerolidol. In protein extracts of spider mite-infested and JA-treated lima bean and cucumber leaves, nerolidol synthase activity was induced (Bouwmeester et al. 1999). However, no TMTT was emitted by JA-treated lima bean leaves (Hopke et al. 1994), indicating a different regulation for TMTT and DMNT emission in lima bean.

In conclusion, we propose that *LeGGPS1* is responsible for provision of precursors for TMTT biosynthesis in tomato. This still needs to be confirmed via a transgenic approach. GGPP synthases other than *LeGGPS1* might also contribute to the pool of GGPP for TMTT biosynthesis. Furthermore we showed that regulation of this gene was dependent on both JA and SA and that GL synthase activity was only induced by JA. Thus we have identified two novel regulatory steps in the biosynthesis of TMTT in tomato. Kant et al. (2004) have previously shown that *DOXPS*, the limiting step in plastidial IPP and DMAPP biosynthesis (Botella-Pavia et al. 2004), was upregulated in tomato by spider mite-feeding (Kant et al. 2004). This brings on the question why there are multiple steps in the biosynthesis of TMTT that are regulated. An explanation for this could be that if GL synthase would be the only regulatory step in TMTT biosynthesis, isoprenoid precursors would soon be depleted when TMTT is formed in the amounts emitted after spider mite-infestation. For example, over expression of phytoene synthase in tomato, thus depleting the GGPP pool, resulted in dwarfed phenotype caused by reduced levels of gibberellins (Fray et al. 1995).

The gene that encodes GL synthase still has to be cloned and characterized. Arabidopsis leaves placed in (*E*)-nerolidol and also untreated transgenic Arabidopsis plants expressing strawberry nerolidol synthase, are capable of producing DMNT (Kappers et al. 2005). This is interesting since DMNT is normally not produced by Arabidopsis whereas TMTT is (Van Poecke et al. 2001). This suggests that the enzymes responsible for the conversion of GL to TMTT are also capable of converting nerolidol to DMNT. The enzymes responsible for these conversions have not yet been identified.

A question that still remains is what the function of *LeGGPS2 in planta* is? Is this GGPP synthase indeed involved in carotenoid biosynthesis in ripening fruit? Another intriguing question that remains unanswered is why SA induces *LeGGPS1*. What is the fate of the GGPP formed after SA-treatment? There is one report on increased chlorophyll and carotenoid content in leaves of Cowpea (*Vigna unguiculata*) after SA-treatment (Chandra and Bhatt 1998). It remains to be determined whether SA-treatment of tomato plants results in a similar increase in chlorophyll and carotenoid content in tomato and that *LeGGPS1* ensures substrate for these products.

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