Metabolic, Genomic, and Biochemical Analyses of Glandular Trichomes from the Wild Tomato Species *Lycopersicon hirsutum* Identify a Key Enzyme in the Biosynthesis of Methylketones

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Medium-length methylketones (C7-C15) are highly effective in protecting plants from numerous pests. We used a biochemical genomics approach to elucidate the pathway leading to synthesis of methylketones in the glandular trichomes of the wild tomato *Lycopersicon hirsutum f glabratum* (accession PI126449). A comparison of gland EST databases from accession PI126449 and a second *L. hirsutum* accession, LA1777, whose glands do not contain methylketones, showed that the expression of genes for fatty acid biosynthesis is elevated in PI126449 glands, suggesting de novo biosynthesis of methylketones. A cDNA abundant in the PI126449 gland EST database but rare in the LA1777 database was similar in sequence to plant esterases. This cDNA, designated *Methylketone Synthase 1* (MKS1), was expressed in *Escherichia coli* and the purified protein used to catalyze in vitro reactions in which C12, C14, and C16 β-ketoacyl–acyl-carrier-proteins (intermediates in fatty acid biosynthesis) were hydrolyzed and decarboxylated to give C11, C13, and C15 methylketones, respectively. Although MKS1 does not contain a classical transit peptide, in vitro import assays showed that it was targeted to the stroma of plastids, where fatty acid biosynthesis occurs. Levels of MKS1 transcript, protein, and enzymatic activity were correlated with levels of methylketones and gland density in a variety of tomato accessions and in different plant organs.

INTRODUCTION

Plants synthesize a multitude of specialized compounds to help ward off pests. Some of these classes of compounds, like terpenes and phenolics, are widely distributed throughout the plant kingdom (Bennet and Wallsgrove, 1994; McGarvey and Croteau, 1995). Others occur sporadically or are limited to one or a few taxa. Medium-length methylketones (Figure 1), found throughout the plant kingdom, are one class of compounds highly effective in protecting plants against pests (Williams et al., 1980; Kennedy, 2003). Early studies on the composition of the essential oils of many species found medium-length methylketones in lime (*Citrus limetta*) leaves (Watts, 1886), in clove (*Eugenia caryophyllus*) and cinnamon (*Cinnamomum zeylanicum*) oil (Walbaum and Huthig, 1902), in palm kernel (*Lodoicea maldivica*), peanut (*Arachis hypogaea*), cottonseed (*Gossypium hirsutum*), and sunflower (*Helianthus annuus*) seed oils (Jasperson and Jones, 1947), and in oil of hop (*Humulus lupulus*) (Sorm et al., 1949). 2-Tridecanone was characterized as a crystalline constituent of the essential oil of matsubasa (*Shizandra nigra maxim*), a plant in the magnolia family, which is used as a bath perfume (Sengoku, 1933). In some plants, the methylke-tone and the derived secondary alcohol are found together, for example, 2-heptanone and 2-heptanol in the oil of cloves (Wehmer, 1931).

Leaves of the wild tomato species *Lycopersicon hirsutum f glabratum* (Williams et al., 1980) are among the most prominent sources of methylketones in plants. Several accessions of this wild species contain mainly the two methylketones 2-undecanone and 2-tridecanone, in concentration ranging between 2700 and 5500 µg per g fresh weight (Antonious, 2001). By comparison, the cultivated tomato *L. esculentum* has only minute amounts of these compounds, up to 80 µg per g (Antonious, 2001). In one of the accessions of the *L. hirsutum f glabratum* (PI134417) the methylketones were reported to compose up to 90% of the tip contents of the glandular trichomes.
Trichomes, both glandular and nonglandular, are prominent features of the foliage and stems in the genus Lycopersicon (Luckwill, 1943), with glandular trichomes predominating on most surfaces and the nonglandular trichomes predominating on leaf veins (Figures 2A and 2B).

The glandular trichomes of various Lycopersicon species have been shown to contain several types of secondary compounds, and when the leaf is touched or chewed by herbivores, the contents of the glands are released onto the leaf surface. Considerable effort has been made to link the presence and density of glandular trichomes to levels of host-plant pest resistance both within and among Lycopersicon species (Carter and Snyder, 1985; Yu et al., 1992; Li et al., 2003). In L. hirsutum, several studies have shown a strong positive correlation among density of leaf glandular trichomes, levels of methylketones, and plant resistance against various pathogens, including tobacco hornworm (Manduca sexta), spotted spider mite (Tetranychus urticae), green peach aphids (Myzus persicae), maize earworm (Heliothis zea), and Colorado potato beetle (Leptinotarsa decemlineata) (Kauffman and Kennedy, 1989; Antonious, 2001).

The prevalence in plants, as well as in other organisms, of methylketones with an odd number of carbons (including the long-chain methylketones [C > 20] found in cuticular waxes) suggests that they are derived from fatty acids, perhaps by decarboxylation of the respective β-ketoacids, which are intermediates in both the biosynthesis and degradation pathways of fatty acids. Several studies in the 1950s through the 1970s investigating the role of microorganisms in the spoilage of fats attempted to elucidate the biosynthesis of methylketones in fungi (Mukherjee, 1951; Forney and Markovetz, 1971). In these experiments, Penicillium was grown in pure culture on individual fatty acids, and in each case the methylketone formed had one fewer carbon than the progenitor fatty acid. This suggested that a decarboxylation reaction was involved, possibly during an abortive fatty acid degradation process. In fatty acid degradation, the intermediates are CoA esters, and free β-ketoacids are known to be highly labile and to undergo spontaneous decarboxylation (Ege, 1989). Thus, the enzyme responsible for the production of 2-tridecanone (in the case of myristic acid) may simply be a thioesterase acting on β-ketomyristoyl-CoA, giving a free β-ketomyristic acid which then decarboxylates spontaneously. But enzymatic decarboxylation could not be ruled out because a purified enzyme was not obtained and, therefore, the

![Figure 1. Structures of Common Methylketones Found in Plants.](image1)

![Figure 2. Glandular Trichomes of Tomato.](image2)

(A) A light microscope picture of the adaxial surface of a young leaf, showing the predominant four-celled glandular trichomes (in focus) and the less abundant long, nonglandular trichomes.

(B) Scanning electron micrograph of the abaxial surface of a mature leaf of L. hirsutum. The four-celled glandular trichomes (arrow and in inset) predominate.

(C) Light micrograph of the four-celled glandular trichomes isolated from L. hirsutum leaves after final purification.
nature of the reaction was not defined further in vitro (Hwang et al., 1976).

Whereas in fungi the methylketones appear to be derived from degradation of fatty acids through a β-ketoacyl-CoA intermediate (Forney and Markovetz, 1971), their synthesis in plants may be equally likely to be derived from a β-ketoacyl-acyl-carrier-protein (ACP) intermediate formed during fatty acid biosynthesis. Under this hypothesis, an esterase could cleave β-ketoacyl-ACP, rather than β-ketoacyl-CoA, to form the unstable free β-ketoacid, which will then decarboxylate, either enzymatically or nonenzymatically to form the methylketone. In this study, we used a combination of chemical, biochemical, and genomics approaches to show that methylketones are not only stored but also synthesized through the biosynthetic pathway of fatty acids in tomato glandular trichomes. We were further able to demonstrate the activity of a novel enzyme, Methylketone Synthase 1 (MKS1) that catalyzes the final step of methylketone synthesis.

RESULTS

Localization of Methylketone Storage to the Glandular Trichomes of L. hirsutum f glabratum

Although the presence of methylketones has been detected in several tomato species, including the cultivated tomato L. esculentum, the highest levels have been reported in L. hirsutum f glabratum, including accessions PI126449, PI134417, and LA0407 (Antonious, 2001). We extracted volatiles from intact leaves of several L. hirsutum accessions (see Methods) and confirmed that L. hirsutum f glabratum accession PI126449 had high levels of the methylketones 2-undecanone and 2-tridecanone, as well as 2-pentadecanone (Table 1). We also analyzed the volatile content in the related L. hirsutum accession LA1777 (Table 1), the only L. hirsutum accession for which a gland EST database was available at the beginning of our investigation. LA1777 was found not to make detectable amounts of methylketones (Table 1). Leaves from both lines were shown to contain a variety of terpenes, mostly sesquiterpenes, in agreement with a previous study on the biosynthesis of terpenes in these accessions (van Der Hoeven et al., 2000).

In L. hirsutum, the predominant type of trichome is a four-celled gland mounted on a relatively short stalk (Figures 2A and 2B). A previous study showed that material extracted directly from attached glands of this type contains methylketones (Lin et al., 1987). Therefore, we tested the correlation between the presence of these glands and methylketone content in different organs of methylketone-producing plants. Methylketone content per unit fresh weight was highest in young leaves, whereas mature leaves contained approximately half this amount (Figure 3A). Glandular trichomes were observed on both sides of the leaves, and young and mature leaves had similar weight to surface ratios, but the density of the glandular trichomes in young leaves (58.8 ± 8.0 glands/mm²) was twice that of mature leaves (31.6 ± 3.7 glands/mm²). Sepals were similar to young and old leaves in having glandular trichomes on both sides and similar weight/surface ratios, but sepal gland density was similar to young leaves, and methylketone content per unit weight was intermediate between that of young and mature leaves (Figure 3A). Petals contained ~20% of the methylketone content per unit weight present in young leaves. Examination of the petal surface revealed that glandular trichomes were present only on the abaxial side around the base of the petal, where cells appeared to contain chlorophyll as well as yellow pigment. Young stems had <7% of the methylketone content per unit weight present in young leaves, and this correlated with the >25-fold reduction in weight/surface ratio of the stem, as well as a lower density of glandular trichomes (15.2 ± 0.9 glands/mm²). We also examined roots, which contained no methylketones (Figure 3A).

To examine more closely the correlation between glandular trichomes and methylketones, we compared leaves and stems for methylketone content before and after these organs were brushed to remove the surface glands. As was observed with other plants containing glands (Gang et al., 2001), it was not possible to remove all glands from leaves by brushing because

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<th>Table 1. Comparison of the Volatile Content in Mature and Young (&lt;2 cm) Leaves of L. hirsutum (LA1777) and L. hirsutum f glabratum (PI126449)a</th>
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<td>2-Undecanone</td>
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<td>2-Tridecanone</td>
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<td>2-Pentadecanone</td>
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a Results are presented as micrograms/gram fresh weight and are the average of four replicates. The less volatile sesquiterpene carboxylic acids were not scored.

b ND, not detected.
such treatment eventually led to excessive damage to the leaves. Leaves after moderate brushing contained only 36% of the amount of methylketones found in unbrushed leaves (Figure 3B). Stems are much harder than leaves, and rigorous brushing of stems led to the removal of almost all glands and 98% of the methylketones (Figure 3B).

We next isolated glandular trichomes from *L. hirsutum* accessions PI126449 and LA1777 (Figure 2B) and examined them for the presence of methylketones. After gland isolation (Figure 2C), volatiles were extracted and analyzed by gas chromatography–mass spectrometry (GC-MS). The glands of LA1777 contained mostly sesquiterpenes and one monoterpenene (ocimene), but no methylketones could be detected (Figure 3C). In PI126449, on the other hand, 2-undecanone, 2-tridecanone, and 2-pentadecanone were prominent peaks (Figure 3C) found in similar ratios to those found in extracts from whole leaves. Moreover, additional peaks tentatively identified by mass spectrometry as modified methylketones were also observed. These results confirm that the glandular trichomes are the site of storage of methylketones.

**Figure 3.** Localization of Methylketones in Glandular Trichomes of Tomato.  
(A) Relative levels of methylketone content in different tissues of the plant, per unit fresh weight. YL, young leaf; ML, mature leaf; ST, stem; RT, root; SE, sepal; PE, petal. Values are shown as percentages of the amount in young leaves. Each value in the graph is an average of three replicates.  
(B) Comparison of the relative levels of methylketones in leaves and stems with or without a brushing treatment to remove glands, per unit fresh weight. Leaf volatiles were extracted from opposite leaflets of the same leaf. One leaflet was extracted directly with methyl-tert-butyl ether after weighing (Brushed −), and the other was brushed under liquid N₂ (Brushed +) before weighing and extraction. Stem samples were cut in half and one half was extracted directly after weighing (Brushed −), and the other was brushed under liquid N₂ (Brushed +) before extraction. For each organ, the level of methylketones in unbrushed leaves was set at 100%. Each value in the graph is an average of three replicates.  
(C) Comparison of the volatile content in the glandular trichomes of *L. hirsutum f. glabratum* (PI126449; top) and *L. hirsutum* (LA1777; bottom). Glands were isolated from young leaves, and the volatile content was extracted with hexane and injected into a gas chromatograph–mass spectrometer. A portion of the gas chromatograph, from 12.5 to 25 min, is shown. Ter, terpene; MKx, unidentified methylketone derivative; 2UD, 2-undecanone; 2TD, 2-tridecanone; 2PD, 2-pentadecanone. All the major peaks in the LA1777 chromatogram were identified as terpenes, mostly sesquiterpenes. The scale at the y axis is in arbitrary units.

**A Gland EST Database of the Methylketone-Producing Accession PI126449 Has High Levels of Sequences Encoding Fatty Acid Biosynthetic Enzymes Compared with an EST Database from LA1777 Glands**

To examine whether the methylketones in *L. hirsutum f. glabratum* are derived from fatty acid biosynthesis or degradation, we constructed an EST database from the glands of PI126449. The prevalence of cDNAs encoding enzymes involved in fatty acid biosynthesis and degradation was determined in the analyzed sequences and compared with those found in the previously reported EST database from trichomes of LA1777 (The Institute for Genomic Research, http://www.tigr.org/tdb/igi/). The PI126449 database contains ~5500 ESTs, and the LA1777 database contains ~2500. Most of the plant genes encoding both the enzymes of the degradative pathway (which occurs in the peroxisomes) and the enzymes of the biosynthetic pathway (which occurs in the plastids) have been molecularly characterized (Mekhedov et al., 2000; Graham and Eastmond, 2002), so this analysis was relatively straightforward. One of the most striking results of this analysis was the high abundance of the cDNA for the ACP in the PI126449 EST database. ACP is a small (~9.5 kD) acidic protein that functions as an essential cofactor in fatty acid synthesis, acyl-ACP desaturation (McKeon and Stumpf, 1982), and plastidic acyl-transferase reactions (Frentzen et al., 1983). Multiple ACP isoforms have been found in evolutionarily diverse species of higher and lower plants, and some are constitutively expressed, while others show developmental and tissue-specific expression (Suh et al., 1999). We identified two distinct contigs in PI126449 that encode two isoforms of the protein (in a 3:1 ratio), which together account for 0.9% of the total transcripts in the glands. The LA1777 EST database, on the other hand, contained only one cDNA (accession number AW616614; 0.04% of total cDNAs) encoding ACP (Figure 4A).

With two exceptions, cDNAs for all other enzymes of the biosynthetic pathway were highly represented in the PI126449
database compared with their representation in the LA1777 database (Figure 4A). The two exceptions are cDNAs for the \( \beta \)-hydroxyacyl-ACP dehydratase (no cDNA in PI126449; 0.04% in LA1777) and enoyl-ACP reductase (0.05% in PI126449; 0.08% in LA1777).

The prevalence of cDNAs encoding the degradative enzymes in the gland EST databases of the two \( L. \) \textit{hirsutum} accessions was, on average, severalfold lower than the prevalence of the cDNAs for the biosynthetic enzymes, and there was no statistically significant difference in their abundance between the two accessions (Figure 4B). Thus, this analysis suggested that the methylketones in PI126449 are synthesized during de novo fatty acid synthesis rather than via the degradative pathway.

**An EST With Homology to Some Esterases Is HighlyExpressed in PI126449 Glands and the Encoded Protein Is Correlated with Methylketone Production**

The most abundant unknown sequence in the PI126449 database (107 ESTs, 2% of total ESTs, ranked third in abundance after a putative secretory carrier membrane protein and \( S \)-adenosyl-\( L \)-homocysteine synthase) encodes a 29-kD protein, with an open reading frame (ORF) containing 265 amino acids (Figure 5). The correct ORF was established by examining several independent full-length cDNAs, two of which contain 182 nucleotides upstream of the initiating ATG (accession number AY701574). The correct ORF was established by examining several independent full-length cDNAs, two of which contain 182 nucleotides upstream of the initiating ATG (accession number AY701574).

There are four stop codons in frame in this nontranslated region, with the 3' most being located 36 nucleotides upstream of the initiating ATG. Two more cDNAs contain nontranslated sequences that include the stop codon proximal to the initiating ATG. In addition, the genomic sequence of this gene is completely identical to the corresponding sequences of all these cDNAs (E. Fridman and E. Pichersky, unpublished data).

The protein encoded by this ORF, which we designated MKS1 (for Methylketone Synthase 1, see below), has 48% sequence identity with tomato methyl jasmonate esterase (LeMJE) (Stuhlfelder et al., 2004) and 44% with the alkaloid biosynthetic enzyme polyneuridine aldehyde esterase (PNAE) from \textit{Rauvolfia serpentina} (Dogru et al., 2000) (Figure 5). In addition, this peptide is similar to several proteins with no proven function, all

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**Figure 4. In Silico Analysis of the \( L. \) \textit{hirsutum} Leaf Glandular Trichome EST Databases.**

**A** Analysis of the enzymes of the fatty acid biosynthesis pathway. The frequencies of specific cDNAs encoding each enzyme (labeled A to F) and the ACP are in parentheses (number per 10,000 ESTs; the number to the left of the diagonal is for PI126449; the number to the right is for LA1777). A, acetyl-CoA carboxylase; B, malonyl-CoA-ACP transacylase; C, \( \beta \)-ketoacyl-ACP synthase I; CIII, \( \beta \)-ketoacyl-ACP synthase III; D, \( \beta \)-ketoacyl-ACP reductase; E, \( \beta \)-hydroxyacyl-ACP dehydratase; F, enoyl-ACP reductase. The total number of ESTs in accession PI126449 is \( \sim 5500 \), and the total number of ESTs in accession LA1777 is \( \sim 2500 \).

**B** Analysis of the peroxisomal core enzymes of the \( \beta \)-oxidation of saturated fatty acids. The frequencies of ESTs (number per 10,000 ESTs) encoding each enzyme (labeled A to C) are in parentheses (left number, PI126449; right number, LA1777). A, acyl-CoA oxidase; B, multifunctional protein, with \( \beta \)-2-trans-enedoyl-CoA hydratase and \( \beta \)-L-hydroxyacyl-CoA dehydrogenase; C, \( \beta \)-ketoacyl-CoA thiolase.
considered to be part of the large α/β-hydrolase fold proteins (Wasi et al., 1998; Zhong et al., 2001; Marshall et al., 2003). This cDNA was represented only once (0.04%; accession number AW617364) in the LA1777 gland EST database. The PI126449 EST database did not contain any other abundant sequences (>0.5%) that encoded proteins with similarity to known thioesterases or other general esterases.

To investigate the correlation between levels of MKS1 and methylketone content, we first sampled young leaves of 10 different accessions of Lycopersicon. We determined their methylketone content, we first sampled young leaves of 10 different accessions of Lycopersicon. We determined their methylketone content by GC-MS and analyzed the transcript levels and methylketone content (Figure 6B). On the other hand, MKS1 transcripts or MKS1 protein were not detected in leaves from the cultivated tomato M82 (L. esculentum; lane 1), from two accessions of L. pennellii (lanes 2 and 3), and from L. hirsutum LA1777 (Figures 6A and 6B, lane 6), all lines that did not contain detectable levels of methylketones (Figure 6C). L. hirsutum f glabratum accession PI251305 and L. hirsutum accession PI127826 had low levels of MKS1 transcripts and MKS1 protein (Figures 6A and 6B, lanes 7 and 4, respectively); metabolic profiling of these two accessions showed that the former had barely detectable levels of methylketones, whereas no methylketones could be detected in the latter (Figure 6C).

The signal was significantly stronger when RNA from purified PI126449 glands was used and still absent with RNA from purified LA1777 glands (Figure 6A, lanes 11 and 12, respectively). Moreover, the high and specific expression of MKS1 in the PI12449 glands, but not LA1777 glands, was also reflected in the abundance of its protein product in the PI12449 glands, but not LA1777 glands (Figure 6B). Using a calibration curve with known amounts of purified MKS1 produced in Escherichia coli, we calculated the concentration of MKS1 in the PI126449 accession to be 2 and 25 ng μg⁻¹ total soluble protein of the leaves and glands, respectively, and 0.5 and 50 ng mg fresh weight⁻¹ of the leaves and glands, respectively.

To further examine the correlation among MKS1, methylketones, and glands, we measured the concentration of MKS1 in different parts of the plant (Figure 7A). MKS1 protein levels were quantified by SDS-PAGE, followed by immunoblotting of the protein samples together with known amounts of the purified MKS1 protein. MKS1 levels were highest in both young and mature leaves and in sepals and lower in stems and petals, generally correlating with the methylketone content of these organs (Figure 3A). As with methylketone level, MKS1 levels were also correlated with the presence of glands because brushed leaves and stems contained much reduced amounts of MKS1 (75 and 90% reduction in the brushed leaves and stems, respectively) (Figure 7B).

### Table 5. Amino Acid Sequence Alignment Comparing the L. hirsutum f glabratum Accession PI126449 MKS1 Protein with Two Functionally Characterized Esterases Belonging to the α/β-Hydrolase Superfamily.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession Number</th>
<th>Amino Acid Sequence</th>
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<tr>
<td>MKS1</td>
<td>AY701574</td>
<td>VRDETLFADLGVCYKCI</td>
</tr>
<tr>
<td>LeMJE</td>
<td>AAF22288</td>
<td>NADVDSKPKK</td>
</tr>
<tr>
<td>PNAE</td>
<td>AAS10488</td>
<td>KPEVKKPKK</td>
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The MKS1 accession number is AY701574. LeMJE, methyl jasmonate esterase from L. esculentum (accession number AAS10488); PNAE, polyneuridine aldehyde esterase from R. serpentina (accession number AAF22288). The first amino acid position of the mature PNAE and LeMJE, determined by N-terminal sequencing, is underlined and in bold. When the same amino acid occurs in two or all three proteins in a given position, it is shown in white on a black background.
a standard transit peptide because N-terminal sequencing of the mature proteins showed that they begin from positions 3 and 7, respectively, of the ORFs (Dogru et al., 2000; Stuhlfelder et al., 2002) (Figure 5). The region between positions 12 and 53, which includes amino acids known to be important for the catalytic activity of PNAE, is highly conserved in MKS1 as well as in LeMJE (Figure 5). Furthermore, the protein encoded by the ORF of the MKS1 cDNA has the same number of amino acids in the 5′-end of this conserved domain. In addition, the MKS1 protein from glands, detected by immunoblotting, appears to have the same or very similar \( M_r \) value to that of \( E. coli \)-produced MKS1 (Figure 6B). These observations strongly suggest that, at most, only a few amino acids are removed from the N terminus to produce the mature \( L. hirsutum f. glabratum \) MKS1; therefore, the N-terminal part encoded by the MKS1 ORF is not a typical, cleavable transit peptide. However, since the chemical structure of methylketones and our gene expression data led us to hypothesize that methylketones are derived from intermediates in fatty acid biosynthesis, which occurs in the chloroplast, we tested the ability of MKS1 to import into isolated chloroplasts.

The in vitro–translated, radiolabeled tomato MKS1 protein was incubated with isolated pea (\( P. sativum \) var Little Marvel) chloroplasts and ATP, followed by reisolation of the chloroplasts, incubation with proteases, lysis, fractionation into membrane and soluble components, and SDS-PAGE. The translated protein was found to be imported into the chloroplast (Figure 6B), as deduced from its resistance to proteolytic digestion by both thermolysin and trypsin, compared with the sensitivity of the unprotected MKS1 (treatment of chloroplasts with Triton X-100 before protease treatment makes their membrane permeable to the proteases; Figures 6A and 6B, lanes 7 to 10), and could be recovered from the soluble fraction of the chloroplast.

The resistance of MKS1 to trypsin after import and its recovery in the soluble fraction of the chloroplast indicate that MKS1 is routed to the stroma compartment after crossing the outer and inner envelopes, as trypsin is known to be able to cleave proteins that protrude into the intermembrane space between the inner and outer envelopes (Figure 6C), but not proteins buried in the inner membrane or found in the stroma (Froehlich et al., 2001).

Identical import and protease resistance behavior to that of MKS1, with the exception of a clear transit peptide cleavage, is observed with the mature small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (labeled mSS, for mature small subunit; Figure 6D), a well-established stromal protein (Olsen et al., 2001).
and Keegstra, 1992). Under the conditions of this assay, the Mr of MKS1 did not appear to decrease after import, consistent with the observation that the MKS1 protein synthesized in E. coli from the complete ORF is essentially identical in size to the one extracted from glands (Figure 6B), indicating that MKS1 import into the plastids is accompanied by the removal of either a few amino acids or none at all.

The Tomato MKS1 Protein Catalyzes the Formation of 2-Undecanone, 2-Tridecanone, and 2-Pentadecanone from β-Ketolauroyl-ACP, β-Ketomyristoyl-ACP, and β-Ketopalmitoyl-ACP in the Glands

PNAE is a member of the a/b-hydrolase superfamily (Dogru et al., 2000), and it catalyzes the deesterification of a methylester with a b-keto functionality (Figure 9A). Once the methyl group is removed, the resulting b-ketoacid decarboxylates, although it is not yet known if this reaction is also catalyzed by PNAE or occurs spontaneously either before or after the b-ketoacid leaves the active site of the enzyme. The similarity of the PNAE esterase protein sequence to MKS1 suggested that the latter may be the hypothesized MKS enzyme that deesterifies b-ketoacyl-ACPs to the b-ketoacids and thus leads to the formation of methylketones after decarboxylation (Figure 9B).

To test if MKS1 catalyzes the deesterification and possibly the decarboxylation of b-ketoacyl-ACPs, we expressed its cDNA in E. coli and purified it to near homogeneity (see Supplemental Figure 1 online). Because the predominant L. hirsutum f. glabratum accession PI126449 methylketone is 2-tridecanone, we first synthesized its hypothesized precursor, [3-14C] β-ketomyristoyl-ACP (because the b-ketoacyl-ACP substrates are unstable and not commercially available, we developed a synthesis method to produce such compounds, see Methods and Supplemental Figures 2 and 3 online). The PI126449 gland protein extract and the purified E. coli-produced MKS1 were tested in a reaction containing the synthesized and trichloroacetic acid–purified [3-14C] β-ketomyristoyl-ACP. A crude protein extract of LA1777 glands and BSA were assayed as controls. The extracts from PI126449 glands reproducibly converted this substrate to a product that was identified as 2-tridecanone by comigration

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The Tomato MKS1 Protein Catalyzes the Formation of 2-Undecanone, 2-Tridecanone, and 2-Pentadecanone from β-Ketolauroyl-ACP, β-Ketomyristoyl-ACP, and β-Ketopalmitoyl-ACP in the Glands

PNAE is a member of the a/b-hydrolase superfamily (Dogru et al., 2000), and it catalyzes the deesterification of a methylester with a b-keto functionality (Figure 9A). Once the methyl group is removed, the resulting b-ketoacid decarboxylates, although it is not yet known if this reaction is also catalyzed by PNAE or occurs spontaneously either before or after the b-ketoacid leaves the active site of the enzyme. The similarity of the PNAE esterase protein sequence to MKS1 suggested that the latter may be the hypothesized MKS enzyme that deesterifies b-ketoacyl-ACPs to the b-ketoacids and thus leads to the formation of methylketones after decarboxylation (Figure 9B).

To test if MKS1 catalyzes the deesterification and possibly the decarboxylation of b-ketoacyl-ACPs, we expressed its cDNA in E. coli and purified it to near homogeneity (see Supplemental Figure 1 online). Because the predominant L. hirsutum f. glabratum accession PI126449 methylketone is 2-tridecanone, we first synthesized its hypothesized precursor, [3-14C] β-ketomyristoyl-ACP (because the b-ketoacyl-ACP substrates are unstable and not commercially available, we developed a synthesis method to produce such compounds, see Methods and Supplemental Figures 2 and 3 online). The PI126449 gland protein extract and the purified E. coli-produced MKS1 were tested in a reaction containing the synthesized and trichloroacetic acid–purified [3-14C] β-ketomyristoyl-ACP. A crude protein extract of LA1777 glands and BSA were assayed as controls. The extracts from PI126449 glands reproducibly converted this substrate to a product that was identified as 2-tridecanone by comigration
with an authentic 2-tridecanone standard (Figure 10A, lane 1). This product was also identified as 2-tridecanone by GC-MS analysis (see Supplemental Figure 4 online). Extracts of LA1777 did not produce any 2-tridecanone (Figure 10A, lane 2). The purified MKS1 protein also catalyzed the formation of 2-tridecanone (Figure 10A, lane 3), whereas a control reaction containing BSA produced no 2-tridecanone (Figure 10A, lane 4).

To further examine the correlation between MKS activity and the presence of glands, we compared the levels of MKS enzymatic activity in the glands to that in leaves and stems with and without the brushing treatment. On average, MKS1-specific activity (per milligram of protein) in the leaf and stem was 48 and 32%, respectively, of that of the glands (Figure 10B). Partial removal of the glands from the leaf and stem reduced the levels of MKS1 activity by 79 and 90%, respectively, compared with the activity with the glands (Figure 10B).

\[ \text{b-Ketolauroyl-ACP and b-ketopalmitoyl-ACP} \]

were synthesized in a similar way, and MKS1 was able to convert \( b \)-ketolauroyl-ACP to 2-undecanone and \( b \)-ketopalmitoyl-ACP to 2-pentadecanone. We next determined the apparent \( K_m \) values of purified MKS1 for all three substrates (Figure 11). The enzyme had an apparent \( K_m \) value for \( b \)-ketomyristoyl-ACP of 3.1 \( \mu M \) (Figure 11A). The apparent \( K_m \) values for \( b \)-ketolauroyl-ACP and \( b \)-ketopalmitoyl-ACP were 1.2 and 10.7 \( \mu M \), respectively.

![Figure 9. The Hypothesized Reaction Leading to Methylketones Is Similar to the Reaction Catalyzed by PNAE.](image)

(A) General scheme of the reaction catalyzed by PNAE. (B) Proposed scheme of the reaction catalyzed by MKS.

![Figure 10. Radio–Thin Layer Chromatography Analysis of the Hexane-Soluble Products from Enzymatic Assays Using [3-14C]β-Ketomyristoyl-ACP as a Substrate.](image)

(A) Enzymatic assays with gland protein and purified MKS1. Arrows indicate the positions of the 2-tridecanone (2TD) standard, the [1-14C] lauric acid standard (C12:0), and the origin. Lane 1, crude protein extract of PI126449 glands; lane 2, crude protein extract of LA1777 glands; lane 3, MKS1 purified from E. coli; lane 4, BSA control. The concentration of the substrate in each reaction was 10 \( \mu M \). Lanes 1 and 2 contained 5 \( \mu g \) total protein. Lanes 3 and 4 contained 0.5 \( \mu g \) of protein. (B) Comparisons of the relative levels of MKS1 activity in stems and leaves with and without treatment to remove glands. Leaves and stems treated as in Figure 3B. The concentration of the substrate and the amount of protein in each reaction was 3 \( \mu M \) and 3 \( \mu g \), respectively. A sample thin layer chromatography plate is shown above, and the bars represent averages of three replicates.
The V_{max} value for the reaction of MKS1 with [3-14C] \( \beta \)-ketomyristoyl-ACP was \( 10^{-8} \) μmoles·s\(^{-1}·\mu\)g protein\(^{-1} \), and similar values were obtained for the reaction with \( \beta \)-ketoauroyl-ACP and \( \beta \)-ketopalmitoyl-ACP.

We also tested the esterase activity of MKS1 with C10:0-ACP, C12:0-ACP, C14:0-ACP, and C16:0-ACP. MKS1 had no esterase activity with these substrates, suggesting that the enzyme is specific to \( \beta \)-ketoacyl-ACP rather than the straight acyl-ACP. The enzyme also did not hydrolyze benzoyl-CoA or the artificial substrates \( p \)-nitrophenyl-palmitoyl and \( p \)-nitrophenyl-decanoyl to the free benzoic acid and fatty acids, respectively, indicating that the enzyme is not a general esterase.

**DISCUSSION**

**The Machinery to Accumulate and Produce Methylketones from Fatty Acids Is Located in the Glands of L. hirsutum f. glabratum**

The four-celled glandular trichomes on the leaves of the wild tomato *L. hirsutum f. glabratum* had previously been recognized to be the site of accumulation of methylketones (Lin et al., 1987). We show that methylketone content correlates well with the presence and density of glands in leaves as well as in other green parts of the plant (Figure 3A). However, the correlation is not perfect; for example, stems have 7% of the methylketone content per unit fresh weight that young leaves do, but young leaves have almost 100-fold more glands per unit fresh weight. This observation suggests that stem glands contain more methylketones because, perhaps, being older, they have had more time to accumulate them. The correlation of methylketone content with glands was further strengthened by examining leaves and stems from which most glands were removed by brushing. Although it was not possible to remove all glands from the surfaces of the plant organs, the great decrease in methylketone content in brushed leaves and stems, as well as the isolation and purification of glands and the demonstration of their methylketone content, support the conclusion that the glands are the main, and perhaps exclusive, site of methylketone storage in the plant (Figures 3B and 3C).

With the demonstration of the glandular trichomes as the major site of methylketone storage, we applied the single-cell EST database approach to determine if these glands might also be the site of synthesis of methylketones and, if so, to identify the genes and enzymes involved in their biosynthesis. Analysis of EST databases constructed from a single type of cell in

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(A) A representative measurement with [3-14C] \( \beta \)-ketomyristoyl-ACP as the substrate. The apparent \( K_m \) value shown is an average of three independent assays.

(B) A measurement with [3-14C] \( \beta \)-ketoauroyl-ACP as the substrate. This assay gave a similar result to the one shown in (A) and was done only once because of the low availability of substrate (see Methods).

(C) A representative measurement with [3-14C] \( \beta \)-ketopalmitoyl-ACP as the substrate. The apparent \( K_m \) value shown is an average of two independent assays.
conjunction with other types of data, such as metabolic profiling, is a powerful method to identify major biochemical pathways operating in the cell. We have previously used this approach to identify genes important in the phenylpropene and terpene pathways by constructing and comparing EST databases from peltate gland cells of several basil varieties (Gang et al., 2001, 2002; Iijima et al., 2004a, 2004b). Gene identification in these studies was based on prevalence of specific sequences, comparisons with known sequences encoding enzymes of similar function (either in terms of substrates or type of reactions), metabolic profiling of the plant material, and enzymatic assays of candidate proteins (Fridman and Pichersky, 2005). This approach has allowed us to determine that methylketones are made via the de novo fatty acid biosynthetic pathway in the chloroplast and to identify MKS1 as the enzyme responsible for the reaction leading from C_{12}, C_{14}, and C_{16} \beta\text{-ketoacyl-ACPs} to the C_{11}, C_{13}, and C_{15} methylketones, respectively. The levels of MKS1 transcripts and protein are closely correlated with the presence of methylketones in the Lycopersicon genus (Figure 6). Furthermore, the levels of MKS1 transcripts, protein, and MKS enzymatic activity were found to correlate well with the presence of glands throughout the plant (Figures 6, 7, and 10). Taken together, these results indicate that these glands are also the major, and perhaps exclusive, site of methylketone biosynthesis.

### MKS1 Is a Plastid Protein

The results of the chloroplast import experiments indicate that MKS1 is targeted to the soluble stroma compartment of the chloroplast (Figure 8B), in close proximity to the site of fatty acid biosynthesis (Walker and Harwood, 1985). The routing of a protein to the plastid stroma without the cleavage of a typical transit peptide is rare but not unprecedented; Rathinasabapathi et al. (1994) reported that betaine aldehyde dehydrogenase is imported into the chloroplast and is ultimately localized in the stroma without cleavage of an N-terminal peptide. In addition, several inner and outer membrane proteins are known not to contain a cleavable transit peptide (Froehlich et al., 2001; Miras et al., 2002). Although our results indicate that MKS1 is not substantially processed during import (Figures 6B and 8B), we cannot exclude the possibility that a small peptide is removed from MKS1 during its targeting to the stroma.

### The Mechanism of Action of MKS1

Our enzymatic characterization of MKS1 indicated that it can use several \(\beta\)-ketoacyl-ACPs with high affinity to produce the corresponding methylketones. However, the efficiency of the reaction was too low to account for the amount of methylketones made by...
the plant. It can be calculated that only 1% of the observed amount of methylketones in the leaves could be made given the amount of MKS1 in the leaves and the \( V_{\text{max}} \) value of the enzyme determined in the in vitro assays. There may be several explanations for this discrepancy between the in vitro rate of the MKS1-catalyzed reaction and the actual in vivo synthesis of methylketones in the cell. It is likely that the in vitro assay we developed is not yet optimized. The enzyme may encode some cofactors that have not yet been identified. For example, we have found that adding the soluble fraction of an *E. coli* extract increases the efficiency by \( \sim 10 \)-fold (E. Fridman and E. Pichersky, unpublished data). In addition, we used spinach (*Spinacia oleracea*) ACP in our assays, but the enzyme may work faster with *L. hirsutum* ACPs. Several previous kinetic characterizations of fatty acid biosynthetic enzymes (e.g., acetyl-CoA carboxylase and malonyl-CoA:ACP transacylase) and polyketide synthases, some of which use substrates containing ACP moieties, have reported either lower in vitro \( V_{\text{max}} \) values or higher \( K_m \) values (Alban et al., 1994) that could not explain the observed rate of polyketide or fatty acid synthesis in the organism. In some cases, the ACP moiety was directly tied to the low \( V_{\text{max}} \) values (Tang et al., 2003), but in other cases the reasons remained undetermined or were attributed to a lack of components of a multienzyme complex, where the product of one reaction is generated immediately adjacent to the active site of the next enzyme in the reaction sequence (e.g., substrate channeling) (Roughan, 1997; Tang et al., 2003).

There are some indications that the plastidic enzymes involved in fatty acid biosynthesis are part of a complex (Roughan and Ohlrogge, 1996). It is also likely that MKS1 is part of this protein complex in the *L. hirsutum* glands and that its kinetic parameters are quite different when it acts as part of such a complex. The existence of such a complex may also relate to the intriguing question of how the substrates of MKS1, which are intermediates in the fatty acid biosynthetic pathway, become accessible to MKS1 and why in *L. hirsutum f. glabratum* the main products are 2-tridecanone, 2-undecanone, and 2-pentadecanone (and not smaller or larger methylketones). Furthermore, although MKS1 showed similar specificity to the three different \( \beta \)-ketoacyl-ACPs that lead to the formation of 2-undecanone, 2-tridecanone, and 2-pentadecanone (Figure 11), the three methylketones exist in different quantities in the glands (Table 1, Figure 3C). It is interesting that in one of the *L. hirsutum f. glabratum* accessions, LA0407, the ratio between 2-tridecanone and 2-undecanone is the opposite of that found in the rest of this subspecies, with 2-undecanone being the major methylketone in the leaf (Antonious, 2001). Yet, the sequence of the LA0407 allele of MKS1 is identical to that of MKS1 from P1126449 (E. Fridman and E. Pichersky, unpublished data). This suggests that the variation in the abundance of the different methylketones in the glands (and the real turnover rate of the enzyme) may be determined by the availability of the substrate rather than the specificity of MKS1 for the different substrates.

**Relationship of MKS1 to Other \( \alpha/\beta \)-Hydrolase Enzymes**

MKS1 is part of the \( \alpha/\beta \)-hydrolase superfamily that includes several enzymes with a proven esterase function. These include, in addition to PNAE and tomato MJE, potato (*Solanum tuberosum*) MJE (accession number AY684102) and PIR7, a protein from rice (*Oryza sativa*) that was shown to have esterase activity with artificial substrates (Waspi et al., 1998). In addition, the tobacco (*Nicotiana tabacum*) SABP2 protein, which has recently been shown to be methyl salicylate esterase (Forouhar et al., 2005), and an uncharacterized protein encoded by an ethylene-induced citrus EST (Zhong et al., 2001), as well as 20 proteins encoded in the Arabidopsis genome, show similarity to MKS1 (Figure 12). PIR7, PNAE, SABP2, and MJE all appear to have defense-related functions. For example, the defense-related rice *Pir7* transcripts were found to accumulate upon infiltration with the resistance-inducing *Pseudomonas syringae pv syringae* (Waspi et al., 1998), and PNAE is involved in the biosynthesis of defense alkaloids in the Indian medicinal plant *R. serpentina*. MJE may be involved in the jasmonate signal transduction pathway that turns on many defense genes (Sasaki et al., 2001; Stuhlfelder et al., 2004). The role of MKS1 in the biosynthesis of the highly protective methylketone is therefore not surprising. However, the origin of MKS1 during evolution and the prevalence of MKS1-related sequences in the plant kingdom (and elsewhere) remain to be determined.

**METHODS**

**Plant Material**

Seeds for the different tomato (Lycopersicon) accessions were obtained from the Tomato Seedstock Center at the University of California (Davis, CA) (LA accessions) and from the USDA–Agricultural Research Service (Ithaca, NY) (PI accessions). Seeds were germinated in the dark for 3 d at 25°C, after which time they were transferred to a growth chamber for 3 weeks at 25°C with 18 h of light. Three-week-old plants were transplanted to pots and arranged in a randomized block design in the greenhouse at 25°C with 18 h of light. For gland extractions, plants were propagated by rooting cuttings, using the rooting powder Hormorex (0.10% indole-3-butyric acid; Brooker Chemical, Chatsworth, CA). Cuttings were rooted under a plastic bag at 25°C with 18 h of light for 2 weeks, after which time plants were transplanted to 1-liter pots containing Sunshine Mix 1 (Sun Gro Horticulture, Bellevue, WA) potting soil at 25°C with 18 h of light.

**Scanning Electron Microscopy**

Young leaves of *L. hirsutum f. glabratum* were prepared for scanning electron microscopy as previously described (Gang et al., 2001).

**Leaf Gland Trichome Isolation and Removal for Protein Extraction, Volatile Analysis, and RNA Isolation**

Leaf glandular trichomes were isolated from young leaves of different tomato accessions following the procedure described previously (Gang et al., 2001) with a few modifications. The glands were collected on a final 40-μm mesh cloth, and the yield was \( \sim 1 \) to 1.5 mL of packed glands per 15 g of leaf sample. Glands were left on ice for 15 min to settle down and were used either for analysis of essential oil constituents or for protein extraction. The crude protein extract of the glands was obtained by resuspending the packed glands in ice-cold gland lysis buffer (10 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM β-mercaptoethanol, and 10% glycerol) followed by sonication for 2 min and collection of the
supernatant after centrifugation at 14,000g at 4°C for 20 min. For RNA isolation, the glands were isolated in a similar way, except that aurintricarboxylic acid (1 mM) was added to all buffers and solutions. After letting the glands settle down on ice for 15 min, the overlying gland isolation buffer was removed and 450 µL of buffer RLT (Qiagen, Valencia, CA) was added for each 100 mg of glands. The glands were lysed by a 30-s sonication pulse, and the total RNA was purified using the RNeasy plant mini kit (Qiagen).

Leaf and stem samples were collected from five independent PI126449 plants that were randomly planted in pots with other tomato accessions. Partial removal of the glands from leaves and stems was performed in liquid N2 with a painter’s brush before weight measurements and extractions. Compared leaves, with or without treatment, were opposite leaflets on the same leaf, and compared stem samples were from the same stem.

cDNA Library Construction, Sequencing, and EST Analysis

A directional cDNA library was constructed as previously described (Gang et al., 2001), and recombinant plasmids from 3840 colonies (10 × 384-well plates) were randomly and automatically isolated and sequenced from both 5’- and 3’-ends, using the T3 and T7 primers, respectively. After removal of vector and poor quality sequences, the remaining 5496 EST sequences were compared against the SWISS-PRO and nonredundant databases using the tblastn search algorithms. The contig assemblies and the singletons were assigned specific functions based on highest similarity and each was BLAST searched against the L. hirsutum (accession LA1777) trichome EST database (The Institute for Genomic Research, http://www.tigr.org/tdb/igl/ to obtain the number of ESTs for each gene.

GC-MS Analysis of Volatiles

Plant samples were weighted and then placed in 5-mL glass vials containing 1 mL of methyl-t-butyl ether. The vials were sealed with rubber septa cups and mildly shaken for 2 h. Toluene was added as an internal standard (0.03%), and the resulting extract was used for GC-MS analysis. GC-MS was performed as described previously (Gang et al., 2001). Three microliters of the extract, with no prior concentration, were loaded on the GC-MS instrument, which was purged with nitrogen. The identity of the main methylketones and terpenes were verified with commercially available standards. For determination of the volatiles in the glands, 100 mg of packed glands (see extraction protocol above) were extracted twice with 200 µL of hexane, followed by concentration and GC-MS analysis.

Chloroplast Import Assays

Pea plants (Pisum sativum var Little Marvel; Olds Seed, Madison, WI) were grown under natural light in the greenhouse at 18 to 20°C. Chloroplasts were isolated from 8- to 12-d-old plants as described previously (Perry and Keegstra, 1994). MKS1, AR6 (Vitha et al., 2003), and RBCS (Olsen and Keegstra, 1992) genes were transcribed, translated, and labeled with [35S]-Met using the TNT-coupled Reticulocyte Lysate system (Promega, Madison, WI). Import assays were performed in 450 µL of import buffer (50 mM Hepes-KOH, pH 8.0; 330 mM sorbitol) containing 75 µL of chloroplasts, 4 mM Mg-ATP, and 5 × 106 dpm precursor protein for 30 min, at room temperature, in the presence of light. Chloroplasts were recovered by sedimentation through a 40% Percoll cushion and then incubated with (+) or without (−) thermolysin or trypsin for 30 min at 4°C as previously described (Jackson et al., 1998), with or without Triton X-100. Intact chloroplasts not treated with Triton X-100 were again recovered by centrifugation through a 40% Percoll cushion then lysed in a solution containing 25 mM Hepes, pH 8.0, and 5 mM EDTA and fractionated into stroma and total membrane (including outer envelope, inner envelope, and thylakoid). Samples treated with Triton X-100 were centrifuged at 100,000g and separated to pellet and supernatant fractions. All import reactions were analyzed by SDS-PAGE. After electrophoresis, the gels were subjected to fluorography and exposed to x-ray film (Eastman-Kodak, Rochester, NY).

RNA Extraction and Gel Blot Analysis

RNA from glands was isolated using the RNeasy plant mini kit (Qiagen) as described above, and leaf RNA was isolated using TRIzol reagent (Gibco-BRL, Grand Island, NY) according to the manufacturer’s protocol. RNA gel blots were performed as described previously (D’Auria et al., 2002) using 2 and 5 µg of RNA in each lane for glands and leaf samples, respectively. An MKS1 probe was synthesized with the rediprime II kit (Amersham Pharmacia Biotech, Piscataway, NJ) using a PCR-amplified fragment of MKS1.

Protein Expression and Purification

The following primers were used to amplify and clone the full ORF of MKS1 from a PI126449 leaf cDNA into the Escherichia coli expression TA cloning vector (pCRT7/CT TOPO-TA; Invitrogen, Carlsbad, CA): forward, 5’-AATGGGAGAAAAGCATGTCGC-3’; reverse, 5’-GCTTATATTAC- TTGTTAGC-3’. PCR conditions were 30 s at 94°C, followed by 25 cycles of 20 s at 94°C, 20 s at 56°C, 40 s at 68°C, and an additional 10 min at 68°C. The full-length MKS1 construct was transformed into BL21(DE3) Codon+ cells, and a single transformed colony was grown at 37°C overnight in 2 mL of LB media containing 100 and 34 µg/mL of ampicillin and chloramphenicol, respectively. The culture was then used to start a 200-mL culture (with the same antibiotics) that was grown at 37°C until an A600 value of 0.4 was obtained. After induction with 0.5 mM isopropyl-1-thio-β-galactopyranoside, the culture was grown overnight at 18°C. Cells were pelleted, harvested, and resuspended in 20 mL of lysis buffer (10 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM β-mercaptoethanol, and 10% [v/v] glycerol). After sonication for 2 min and centrifugation at 14,000g for 20 min at 4°C, the supernatant was collected and kept at −80°C for further applications.

A plasmid containing the spinach (Spinacia oleracea) ACP isoform I gene using codons preferred by E. coli, with a C-terminal His-tag (pSACP-1a, Broadwater and Fox, 1999), was kindly provided to us by Brian Fox (University of Wisconsin, Madison). The plasmid was transformed into BL21(DE3) LysS cells, and a single transformed colony was grown at 37°C overnight in 20 mL of LB medium containing 100 and 34 µg/mL of ampicillin and chloramphenicol, respectively. The culture was then used to start a 500-mL terrific-broth culture (Tartof and Hobbs, 1987) (with the same antibiotics) that was grown at 37°C until an A600 value of 1.0 was obtained. After induction with 0.5 mM isopropyl-1-thio-β-galactopyranoside, the culture was grown overnight at 18°C. Cells were pelleted, harvested, and resuspended in 50 mL of ACP lysis buffer (20 mM Tris-HCl, pH 7.9, 250 mM NaCl, 10 mM imidazole, 1% Tween 20, 10 mM β-mercaptoethanol, and 10% [v/v] glycerol). The lysate was stirred on ice for 1 h with 50 µg/mL of lysozyme and DNase (Sigma-Aldrich, St. Louis, MO). After sonication for 2 min and centrifugation at 30,000g for 20 min at 4°C, the supernatant was collected and kept at −80°C until purification.

MKS1 was purified from E. coli cell lysates by diethylaminoethyl-cellulose anion exchange column chromatography (DE53, Sigma-Aldrich) followed by chromatography on a Hi-Trap Phenyl HP (hydrophobic interactions) column (Amersham Pharmacia Biotech) connected to a fast protein liquid chromatography system. MKS1 did not bind to DE53 and eluted with the flow-through, thus achieving a substantial purification. It bound well to a Phenyl HP column, eluting at ~0.3 to 0.35 M.
(NH₄)₂SO₄. Fractions with highest concentrations of MKS1 eluting from the Phenyl HP column were concentrated by centrifugation as described previously (Iijima et al., 2004a).

Spinach ACP was purified from E. coli cell lysates on a column containing 2 mL of packed Ni²⁺-NTA resin (Qiagen). The column was equilibrated with 40 mL of double distilled water, followed by 40 mL of ACP lysis buffer before loading all 50 mL of lysate. The column was then washed once with 20 mL of ACP lysis buffer followed by a 40 mL wash with the same buffer but with no Tween 20. The His-tagged ACP was eluted with 4 mL of ACP lysis buffer with no Tween 20 and with 250 mM imidazole. The eluted 0.5-mL fractions were analyzed on a 15% SDSPAGE, and fractions with high levels of ACP-His6 were pooled and with the same buffer but with no Tween 20. The His-tagged ACP was washed once with 20 mL of ACP lysis buffer followed by a 40 mL wash containing 2 mL of packed Ni²⁺-NTA resin (Qiagen). The column was then equilibrated with 20 volumes (10 mL) of double distilled water, followed by 20 volumes of 10 mM Mes, pH 6.1, with 250 mM imidazole. The eluted 0.5-mL fractions were analyzed on a 15% SDS-PAGE, and fractions with high levels of ACP-His6 were pooled and stored at −80°C for further applications.

The acyl-acyl carrier protein synthase enzyme (Aas) was over-expressed and purified from E. coli cell line BL21(DE3) LysS, using the plasmid and procedures described previously (Shanklin, 2000).

Preparation of MKS1 Antibodies and Protein Gel Blots

One milligram of the purified protein (see Supplemental Figure 1 online, lane 5) was electrophoresed on a 13% SDS-PAGE gel, stained with Coomassie Brilliant Blue, and the protein excised from the gel. Antibodies were prepared by Cocalico Biologicals (Reamstown, PA) by injecting macerated gel fragments containing the purified MKS1 and following the company’s protocol. Specificity of the antibody was tested with the preinjection serum and with different purified proteins (data not shown). Anti-MKS1 antibodies were used at a 1:2000 dilution and incubated with the protein gel blots for 2 to 16 h. All other conditions of the protein gel blots were performed as described previously (Dudareva et al., 1996).

Substrate Preparation, Enzyme Assays, and Product Identification

The coupled enzymatic assay we developed was done in three steps, with the first two steps needed to produce the β-ketoacyl-ACP substrate for the reaction (third step) catalyzed by the putative MKS (see Supplemental Figure 2 online). The following is a description of the synthesis of [3-¹⁴C] β-ketoacyl-CoA synthase I, which condenses the malonyl-ACP and [1-¹⁴C] lauric acid (the starting material in step 1) is reduced to C₁₂ alcohol (see Supplemental Figure 3 online).

In the first step, [1-¹⁴C] 12:0 lauric acid (ARC 257; American Radiochemicals, St. Louis, MO) is converted to an acyl-ACP in a reaction containing spinach ACP and an E. coli acyl-ACP synthetase (Rock and Garwin, 1979). (This E. coli acyl-ACP synthetase couples C₁₂:0 and C₁₄:0 fatty acids efficiently, but it has low coupling efficiency with decanoic acid [C₁₀:0], thereby limiting the amount of [3-¹⁴C] β-lauroyl-ACP that we were ultimately able to make). Because the ACP that was used in these syntheses carried a His-tag, a Ni²⁺-NTA column (0.5 mL; Qiagen) was used to purify the acyl-ACP product. Before loading the samples, the column was equilibrated with 20 volumes (10 mL) of double distilled water, followed by 20 volumes of 10 mM Mes, pH 6.1. After loading the synthesized acyl-ACP, the column was washed with 10 and 20 volumes of 10 mM Mes, pH 6.1, with and without 10 mM imidazole, respectively. The purified substrate was eluted with 2 mL of 10 mM Mes, pH 6.1, with 250 mM imidazole, and the 250-μL fractions were subjected to scintillation counting to detect the active fractions, which were kept at −80°C for further applications.

In the next step, [3-¹⁴C] β-ketoacyl-CoA synthetase I (Rock and Garwin, 1979) was supplied to the MKS1 reaction containing spinach ACP and the [1-¹⁴C] lauric acid-CoA, which converts the supplied malonyl-CoA and ACP to malonyl-ACP, and the β-ketoacyl-CoA synthase I, which condenses the malonyl-ACP and the [1-¹⁴C] lauric acid-CoA to [3-¹⁴C] β-ketoacyl-CoA (Figure 4A). The success of this step is monitored by reacting an aliquot with the reducing agent NaH₄B, followed by radio–thin layer chromatography (TLC) with standards (Garwin et al., 1980). Any [1-¹⁴C] lauroyl-ACP (the product of step 1) that failed to elongate, as well as free [1-¹⁴C] lauric acid (the starting material in step 1) is reduced to C₁₃ alcohol (see Supplemental Figure 3 online), and the product of step 2, [3-¹⁴C] β-ketoacyl-CoA, is reduced to C₁₄, 1,3-diol (see Supplemental Figure 3 online). The substrate was then purified by adding 2.5% trichloroacetic acid, incubating on ice for 10 min, and centrifuging at 14,000g for 5 min, followed by a gradual resuspension of the pellet with 100 mM Tris-HCl to pH 6 to 7.

Sequence Alignment and Construction of the Phylogenetic Tree

Sequence alignment and construction of the phylogenetic tree were performed using ClustalX (version 1.81), and the neighbor-joining method was used with 1000 bootstrap trials. The full actual alignment for the tree is included in Supplemental Figure 5 online. TreeView was used to visualize the resulting tree (Page, 1996). A maximum parsimony tree was also generated using the PAUP* program (Sinauer Associates, Sunderland, MA), and it showed the same branches as those in the neighbor-joining tree.

Access to the Glandular Trichome EST Database

The PI126449 glandular trichome EST database, including a BLAST search engine, can be accessed through our Web site (http://www.biology.lsa.umich.edu/research/labs/pichersky/).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under the following accession numbers: MKS1 from L. hirsutum (accession PI126449), AV701574; MJE from S. tuberosum (potato), AY684102.
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