Heterologous Expression of Methylketone Synthase1 and Methylketone Synthase2 Leads to Production of Methylketones and Myristic Acid in Transgenic Plants

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Some plants produce methylketones as potent defense compounds against various insects. Wild tomato (Solanum habrochaites), a relative of the cultivated tomato (Solanum lycopersicum), synthesizes large amounts of 2-methylketones in its glandular trichomes, but cultivated tomato trichomes contain little or no methylketones. Two enzymes, Solanum habrochaites methylketone synthase1 (ShMKS1) and ShMKS2, are required to convert β-ketoacyl acyl-carrier protein intermediates of the fatty acid biosynthetic pathway to methylketones. ShMKS2 is a thioesterase that hydrolyzes β-ketoacyl acyl-carrier protein, and ShMKS1 is a decarboxylase that converts the resulting 3-ketoacids to 2-methylketones. We introduced ShMKS2 by itself or together with ShMKS1 to Arabidopsis (Arabidopsis thaliana), tobacco (Nicotiana tabacum), and cultivated tomato under the control of the 35S, Rubisco small subunit, and tomato trichome-specific promoters. Young tobacco and Arabidopsis plants expressing both genes under the control of 35S and Rubisco small subunit promoters produced methylketones in their leaves but had serious growth defects. As plants matured, they ceased to produce methylketones. Tobacco plants but not Arabidopsis or tomato plants expressing only ShMKS2 under the 35S promoter also synthesized methylketones, but at a lower rate. Transgenic cultivated tomato plants expressing ShMKS1 and ShMKS2 under trichome-specific promoters had slightly elevated levels of methylketone. Trace amounts of myristic acid were also detected in transgenic plants constitutively expressing ShMKS2 with or without ShMKS1. These results suggest that increases in methylketone production in plants will require the targeting of the pathway to self-contained structures in the plant and may also require increasing the flux of fatty acid biosynthesis.

The wild tomato species Solanum habrochaites ssp glabratum (accession no. PI126449; previously known as Lycopersicon hirsutum ssp glabratum) has long been known for its resistance to a wide spectrum of arthropod pests, including spider mites (Tetranychus urticae), glasshouse whitefly (Trialeurodes vaporariorum), tomato fruitworm (Heliothis zea), Colorado potato beetle (Leptinotarsa decimlineata), tomato pinworm (Keiferia lycopersicella), and tobacco hornworm (Manduca sexta), owing to its production of methylketones (Maluf et al., 1997). Methylketones, mainly in the form of 2-undecanone and 2-tridecanone, are found in prodigious amounts in leaves of wild tomato and exhibit strong pesticidal activity, with 2-tridecanone as the most effective compound (Williams et al., 1980; Antonious et al., 2003). The toxicity of 2-tridecanone to arthropods was confirmed by a positive association of its concentration in vivo in M. sexta or Scrobipalpuloides absoluta with the lethality rate exhibited by these two insects (Ferry and Kennedy, 1987; Gonçalves et al., 1998). Because cultivated tomato (Solanum lycopersicum, previously known as Lycopersicon esculentum) plants possess very low levels of methylketones, and breeding insect-resistant cultivated tomato varieties that accumulate methylketones has not been successful (Hartman and St. Clair, 1998), understanding the biosynthetic mechanism in wild tomato may help to engineer methylketone-based defenses into cultivated tomato plants.

Biosynthesis and storage of methylketones are confined to dedicated structures in wild tomato termed type VI trichomes (Antonious et al., 2003; Fridman et al., 2005). Trichomes are small protrusions of epidermal originated cells on the surface of aerial parts of approximately one third of vascular land plants (Luckwill, 1943; Schilmiller et al., 2008). In the Solanum genus, seven types of trichomes have been reported in various tomato species, with the type VI trichome found in all of those species (Luckwill, 1943; McDowell et al., 2011).

Type VI trichomes in S. habrochaites have a multicell stalk and four gland cells on the apex of the stalk, and are densely present on the surfaces of leaves and stems (Ben-Israel et al., 2009). Methylketones accumulate in large amounts in type VI trichomes, specifically in the apical gland cells, with concentrations of up to 8 mg/g fresh leaf weight (FLW) observed (Fridman et al., 2005). Analysis of a type VI-specific EST database from wild tomato, which is a
methylketone-producing line, followed by gene expression comparison with a trichome EST database from wild tomato (accession no. LA1777), which does not produce methylketones, identified the gene *Solanum habrochaites* methylketone synthase1 (*ShMKS1*) as correlated with methylketone production (Fridman et al., 2005). Additional genetic and genomic analyses identified a second gene, designated *ShMKS2*, whose expression was also positively associated with methylketone production (Ben-Israel et al., 2009).

*ShMKS1* and *ShMKS2* are specifically expressed in the gland cells of type VI trichomes in wild tomato, and both proteins are targeted to the plastids (Yu et al., 2010; Akhtar et al., 2013; G. Yu, A.L. Schilmiller, R.L. Last, and E. Pichersky, unpublished data). In vitro biochemical assays and heterologous expression in *Escherichia coli* demonstrated that the *ShMKS2* protein is a thioesterase that hydrolyzes β-ketoacyl acyl-carrier protein (β-ketoacyl-ACP), primarily C12 and C14 intermediates of the fatty acid biosynthetic pathway, to give the corresponding 3-keto acids, and that *ShMKS1* is a decarboxylase that converts the 3-keto acids to the corresponding C11 and C13 2-methylketones (i.e. 2-undecanone and 2-tridecanone; Yu et al., 2010). The restriction of methylketone biosynthesis and storage to trichomes, where these chemicals can easily come in contact with an attacking herbivore, also has the additional advantage of isolating such toxic compounds from the rest of the plant (Schilmiller et al., 2008). In addition, since the enzymes that make methylketones compete with substrates for fatty acid biosynthesis, synthesis in the trichomes could be a way to avoid competition for substrates in all other plant cells.

Transcripts of *ShMKS1* and *ShMKS2* in wild tomato plants are in 800-fold and 300-fold greater abundance than the levels of the transcripts of the orthologous genes, *SIMKS1a* and *SIMKS2c*, in the cultivated tomato (Fridman et al., 2005; Ben-Israel et al., 2009). Consistent with this observation, methylketones in the trichomes of cultivated tomato plants are present at low and often undetectable levels. However, the *SIMKS1a* and *SIMKS2c* proteins are highly similar to the wild tomato proteins (95% and 91% identical), and expressing the

![Figure 1. Growth phenotypes of transgenic Arabidopsis plants expressing *ShMKS1*, *ShMKS2*, or both genes.](image-url)

**Figure 1.** Growth phenotypes of transgenic Arabidopsis plants expressing *ShMKS1*, *ShMKS2*, or both genes. A to F, Representative pictures of Arabidopsis seedlings grown on plates 8 DAG: wild type (A), p35S::ShMKS2 lines (B and E), p35S::ShMKS2-pRbcs::ShMKS1 lines (C and F), and p35S::ShMKS1 (D). G to N, Pictures of plants 23 DAG (G to I) and 35 DAG (K to N): wild-type (G and K), p35S::ShMKS1 lines (H and L), p35S::ShMKS2 lines (I and M), and p35S::ShMKS2-pRbcs::ShMKS1 lines (J and N). The length of the square pots is 9 cm. True leaves are those with trichomes, as indicated by the arrowheads in the top row.
cultivated tomato SlMKS2 gene in *E. coli* resulted in a similar profile of methylketone production as expressing ShMKS2 (Ben-Israel et al., 2009). It was therefore deemed possible that the extremely low levels of methylketone production in the trichomes of cultivated tomato are caused mainly by the low levels of expression of its MKS1 and MKS2 genes, although other genetic factors have been implicated in enhanced methylketone production in wild tomatoes (Ben-Israel et al., 2009). To test this hypothesis, we introduced ShMKS1 and ShMKS2 genes into cultivated tomato under the control of their own promoters. In addition, to test whether methylketone production could be introduced to non-*Solanum* species and nontrichome tissues, tobacco (*Nicotiana tabacum*) and Arabidopsis (*Arabidopsis thaliana*) plants were transformed with ShMKS1 and ShMKS2 (or both) under the control of constitutively active promoters. Here we report that constitutive expression of the two genes in transgenic tobacco and Arabidopsis, but not in cultivated tomato, resulted in methylketone production. Constitutive expression of ShMKS2, either with or without ShMKS1, in the three plant species tested here led to myristic acid accumulation in planta. However, trichome-specific expression of ShMKS1 and ShMKS2 caused only small increases in concentrations of methylketones in the trichomes of transformed cultivated tomatoes.

**RESULTS**

**Transgenic Arabidopsis Plants Overexpressing ShMKS2, But Not ShMKS1, Exhibit Lesions**

To generate transgenic Arabidopsis lines expressing ShMKS1 or ShMKS2, we spliced these two genes into expression vectors downstream of the cauliflower mosaic virus 35S promoter (designated p35S::ShMKS1 and p35S::ShMKS2, respectively). These vectors were then introduced into wild-type Arabidopsis ecotype Columbia-0 by *Agrobacterium tumefaciens*-mediated transformation. To generate transgenic Arabidopsis lines expressing both ShMKS1 and ShMKS2, the open reading frame (ORF) of ShMKS1 was fused to a *Rubisco small subunit* promoter (designated pRbcs) and added to the p35S::ShMKS2 expression vector. The vector carrying both genes, designated p35S::ShMKS2-pRbcs::ShMKS1, was then transferred into wild-type Arabidopsis (Columbia-0). For all transformants, the presence of intact ORF sequences of ShMKS1 and/or ShMKS2 was verified by genomic DNA PCR. Reverse transcription PCR (RT-PCR) was also performed to confirm the expression of these two genes, and only lines with clear gene expression were chosen for further analysis. Four independent lines of transformants expressing ShMKS1, seven independent lines expressing ShMKS2, and four independent lines expressing both genes were selected. Seeds from the transgenic lines were collected, germinated on kanamycin-containing Murashige and Skoog (MS) plates, and further analyzed.

All of the Arabidopsis plants expressing p35S::ShMKS1 were morphologically indistinguishable from wild-type Arabidopsis (Fig. 1, A, D, G, H, K, and L). On the other hand, both the p35S::ShMKS2 and the p35S::ShMKS2-pRbcs::ShMKS1 expressing lines exhibited growth retardation and leaf lesions by 8 d after germination (DAG). The first pair of true leaves of the p35S::ShMKS2 plants initiated but were not capable of expanding fully to their maturity (Fig. 1, B and E) and they remained colorless, whereas the cotyledons also developed regional chlorosis (Fig. 1, B and E). In plants expressing both ShMKS1 and ShMKS2, the first true leaves developed properly but the cotyledons showed chlorosis before the second pair of true leaves emerged (Fig. 1, C and F).

The majority of seedlings expressing ShMKS2 or both ShMKS1 and ShMKS2 could not survive after transfer to soil, and those that survived grew at a much slower rate than did the wild type or plants expressing ShMKS1 (Fig. 1, G–J). However, the severity of the chlorosis abated as the plants developed, although these plants still had a reduced growth rate (Fig. 1, K–N).

### Table 1. Fatty acid compositions of transgenic and wild-type Arabidopsis plants in early and late developmental stages

Data are presented as the mean ± sd. The sd values were calculated from three biological measurements. *P* values were from a one-tail Student’s *t* test heteroscedastic-type analysis. O, Old plants (35 DAG); N.D., not detected; Y, young plants (8 DAG).

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>C14</th>
<th>C16</th>
<th>C16-3</th>
<th>C18-1</th>
<th>C18-3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type Y</td>
<td>1.13 ± 0.16</td>
<td>1.35 ± 0.31</td>
<td>0.71 ± 0.13</td>
<td>3.77 ± 0.61</td>
<td>6.96 ± 0.66</td>
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</tr>
<tr>
<td>p35S::MK51 Y</td>
<td>1.37 ± 0.03</td>
<td>1.28 ± 0.30</td>
<td>0.80 ± 0.24</td>
<td>4.42 ± 0.60</td>
<td>7.88 ± 0.56</td>
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</tr>
<tr>
<td>p35S::MK2 Y</td>
<td>0.02 ± 0.004</td>
<td>1.56 ± 0.21a</td>
<td>1.77 ± 0.55</td>
<td>0.95 ± 0.14</td>
<td>4.44 ± 0.25</td>
<td>8.74 ± 0.99a</td>
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<tr>
<td>p35S::MK2-pRbcs::MK51 Y</td>
<td>0.17 ± 0.07</td>
<td>2.35 ± 0.31a</td>
<td>1.79 ± 0.71</td>
<td>1.31 ± 0.17b</td>
<td>4.70 ± 0.51a</td>
<td>10.33 ± 1.71b</td>
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<tr>
<td>Wild-type O</td>
<td>0.58 ± 0.10</td>
<td>0.42 ± 0.07</td>
<td>0.39 ± 0.05</td>
<td>1.77 ± 0.04</td>
<td>3.16 ± 0.08</td>
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<tr>
<td>p35S::MK51 O</td>
<td>0.44 ± 0.08</td>
<td>0.36 ± 0.05</td>
<td>0.29 ± 0.11</td>
<td>1.46 ± 0.18a</td>
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<tr>
<td>p35S::MK2 O</td>
<td>0.49 ± 0.13</td>
<td>0.41 ± 0.08</td>
<td>0.33 ± 0.14</td>
<td>1.58 ± 0.41</td>
<td>2.82 ± 0.72</td>
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<tr>
<td>p35S::MK2-pRbcs::MK51 O</td>
<td>0.01 ± 0.004</td>
<td>0.46 ± 0.04</td>
<td>0.35 ± 0.04</td>
<td>0.26 ± 0.07</td>
<td>1.38 ± 0.18</td>
<td>2.45 ± 0.34</td>
</tr>
</tbody>
</table>

*P* < 0.05. *bP* < 0.01.
Production of Myristic Acid and Other Fatty Acids in Transgenic Arabidopsis Overexpressing ShMKS2

Because ShMKS2 is a thioesterase that utilizes fatty acid biosynthetic intermediates and fatty acids are involved in multiple aspects of plant growth (Ohlrogge and Browse, 1995), the observed growth defects of transgenic Arabidopsis overexpressing ShMKS2 are likely a result of interruption of fatty acid biosynthesis. To test this, both free fatty acids and O-acyl lipids from fresh leaves of seedlings at 8 DAG, and from rosette leaves at 35 DAG from various transgenic lines were transmethylated or acid-catalyzed transesterified, and the fatty acid methyl esters were extracted and analyzed by gas chromatography-mass spectrometry (GC-MS). For both transgenic and wild-type plants, higher concentrations of fatty acids per gram FLW were detected at 8 DAG than at 35 DAG (Table I). Plants overexpressing ShMKS1 showed minor differences that were not statistically significant in each fatty acid concentration or in total fatty acid concentration compared with the wild type. Both the p35S::ShMKS2 and p35S::ShMKS2-\textit{pRbs::ShMKS1} lines had statistically significant elevated levels of palmitic acid and myristic acid at 8 DAG, with the lines expressing both
genes having the highest level, and the p35S::ShMKS2-pRbs::ShMKS1 line also had statistically significant elevated levels of C18-1 and C18-3 (Table I). At 35 DAG, trace amounts of myristic acid were detected only in plants overexpressing both ShMKS1 and ShMKS2, and only minor differences in the relative levels of other fatty acids were observed between different plant lines (Table I).

**Production of Methylketones in Transgenic Arabidopsis Plants Expressing Both ShMKS1 and ShMKS2**

Volatile from 8-DAG transgenic seedlings growing on plates were collected by solid phase microextraction fiber and analyzed by GC-MS. Wild-type Arabidopsis seedlings emitted very little volatile compounds (Supplemental Fig. S1A). Three compounds were easily distinguished from lines expressing both ShMKS1 and ShMKS2, but not from plants expressing only ShMKS1 or only ShMKS2, compared with wild-type plants (Fig. 2A). Compounds 1 and 3 were identified as 2-tridecanone and 2-pentadecanone, respectively, because their retention time and mass spectra both matched that of the standards (Fig. 2). Compound 2 had a similar mass spectra profile to those of straight-chain 2-alcohols, but had a retention time of 14.3 min and a M_r of 199, which is between the standards of 2-dodecanol (13.1 min and M_r 185) and 2-tetradecanol (15.3 min and M_r 213; Fig. 2B). Thus, compound 2 was likely to be 2-tridecanol. The methylketone production rate by 8-DAG seedlings expressing both ShMKS1 and ShMKS2 was roughly 1.8 μmol/g FLW in a 5-h period (Table II). However, no methylketone emission could be detected from any transformants after they were transferred to soil (data not shown).

### Table II. The amount of methylketones emitted by transgenic plant seedlings (8 DAG) in a 5-h period

Data are presented as the mean ± sd. The sd values were calculated from three biological measurements. N.D., Not detected.

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>Methylketone Amount (μmol/g FLW/5h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis p35S::ShMKS2-pRbs::ShMKS1</td>
<td>1.08 ± 0.96 0.35 ± 0.16 0.41 ± 0.22 1.83 ± 1.34</td>
</tr>
<tr>
<td>Tobacco p35S::ShMKS2</td>
<td>2.06 ± 0.22 0.36 ± 0.03 0.40 ± 0.05 2.83 ± 0.30</td>
</tr>
<tr>
<td>Tobacco p35S::ShMKS2-pRbs::ShMKS1</td>
<td>0.71 ± 0.03 8.62 ± 1.40 1.58 ± 0.42 2.02 ± 0.46 12.93 ± 2.29</td>
</tr>
</tbody>
</table>

**Analysis of Transgenic Tobacco Plants Overexpressing ShMKS1 and/or ShMKS2**

To further examine whether overexpression of ShMKS1 and ShMKS2 is sufficient to cause methylketone production in other plant species, the same constructs, p35S::ShMKS2-pRbs::ShMKS1 and p35S::ShMKS2, were introduced into wild-type tobacco. p35S::ShMKS1 was not introduced into tobacco because the Arabidopsis lines expressing ShMKS1 showed no detectable changes. Five independent tobacco lines overexpressing both ShMKS1 and ShMKS2 and four lines overexpressing only ShMKS2 were selected. Transgenic tobacco plants with either of the two constructs displayed chlorosis and malformation of the first pair of true leaves when growing on kanamycin-containing MS plates (Fig. 3). Tobacco plants...
expressing both ShMKS1 and ShMKS2 displayed a larger area of chlorosis at 8 DAG and stunted growth at 45 DAG (Fig. 3, A–C and G–I). The first pair of true leaves failed to fully develop by 20 DAG for both constructs (Fig. 3, D–F).

Fatty acids from fresh leaves of 8 DAG seedlings were transmethylated and extracted, and then analyzed by GC-MS. Tobacco lines expressing only ShMKS2 had higher levels of myristic acid per gram FLW than tobacco lines overexpressing both ShMKS1 and ShMKS2 (Table III). Both lines showed a general trend of increases in concentrations of C16, C18, and C18-1 and decreases in C18:2 and C18:3 concentrations, but the differences in only 7 of the 12 pairwise comparisons with wild-type values were statistically significant (Table III).

Volatile analysis of tobacco seedlings expressing both ShMKS1 and ShMKS2 revealed that they emitted methylketones (Fig. 4). In addition to the same three compounds observed in Arabidopsis (2-tridecanone, 2-tridecanol, and 2-pentadecanone), tobacco volatiles contained small amounts of several other compounds (numbered 4 to 8 in Fig. 4A). Compound 4 was identified as 2-undecanone by its mass spectrum (Fig. 4B). Compounds 5 to 8 could not be identified. In contrast with Arabidopsis, in which transgenic plants expressing only ShMKS2 showed no methylketone emission, the tobacco counterpart lines did emit some methylketones (Fig. 4A), with 2-tridecanone, 2-tridecanol, and 2-pentadecanone as the three major compounds. However, the transgenic tobacco plants expressing both genes had a faster methylketone production rate per FLW than the tobacco lines expressing only ShMKS2 (12.93 μmol/g FLW/5 h versus 2.83 μmol/g FLW/5 h; Table II).

The transgenic tobacco plants also suffered a high lethality rate when transferred to soil and displayed growth retardation similar to the Arabidopsis ShMKS2-expressing lines (data not shown). At 45 DAG, tobacco plants expressing only ShMKS2 showed attenuated chlorosis but fully extended leaves, whereas tobacco plants expressing both ShMKS1 and ShMKS2 still displayed severe growth retardation (Fig. 3, G–I).

Production of Transgenic Tomato Plants with Trichome-Specific Expression of ShMKS1 and ShMKS2

We recently showed that the promoter of ShMKS1 is highly active in type VI trichomes of cultivated tomato

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>Wild type N.D.</th>
<th>p35S:ShMKS2 0.45 ± 0.09</th>
<th>p35S:ShMKS2-pRbcS1:ShMKS1 0.17 ± 0.04</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14 Fatty Acids (μmol/g FLW)</td>
<td></td>
<td>1.36 ± 0.24</td>
<td>2.06 ± 0.50</td>
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<tr>
<td>C16</td>
<td>0.27 ± 0.07</td>
<td>0.55 ± 0.03a</td>
<td>0.67 ± 0.14b</td>
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<tr>
<td>C18</td>
<td>0.15 ± 0.03</td>
<td>0.21 ± 0.04</td>
<td>0.54 ± 0.01a</td>
</tr>
<tr>
<td>C18-1</td>
<td>2.36 ± 0.17</td>
<td>1.66 ± 0.26a</td>
<td>3.62 ± 0.36a</td>
</tr>
<tr>
<td>C18-2</td>
<td>6.08 ± 0.52</td>
<td>1.27 ± 0.10a</td>
<td>3.63 ± 0.09a</td>
</tr>
<tr>
<td>C18-3</td>
<td>10.22 ± 0.08</td>
<td>6.21 ± 0.76b</td>
<td>10.67 ± 1.08</td>
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</tbody>
</table>

Table III. Fatty acid compositions of transgenic and wild-type tobacco seedlings (8 DAG)

Data are presented as the mean ± SD. The SD values were calculated from three biological measurements. N.D., Not detected.

*aP < 0.05.  

Figure 4. Chromatography and mass spectra of transgenic tobacco volatiles. A, Shown are chromatographs of a 5-h volatile collection of a tobacco line expressing both ShMKS1 and ShMKS2 and a line expressing only ShMKS2. 2-PD, 2-pentadecanone, 2-TD, 2-tridecanone, 2-TO, 2-tridecanol. B, Mass spectrum of compound 4 in A. Compound 4 was identified as 2-undecanone.
To test the activity of the ShMKS2 promoter in cultivated tomato, we made a construct that fused the 1.5-kb upstream region of ShMKS2 with GFP and transformed cultivated tomato plants. In these transgenic plants, a strong GFP signal was observed in type VI trichomes as well as some weak GFP signals in other types of trichomes (Fig. 5, A–C), indicating that the ShMKS2 promoter is strongly active in these trichomes in the cultivated tomato as well.

Next, we generated transgenic cultivated tomato plants carrying both ShMKS1 and ShMKS2 each under the control of its own promoter using pShMKS2::ShMKS2-pShMKS1::ShMKS1 construct as well as plants carrying only ShMKS2 under the control of its own promoter (pShMKS2::ShMKS2; see “Materials and Methods”). RT-PCR analysis of total RNA from isolated trichomes of transgenic cultivated tomato lines expressing pShMKS2::ShMKS2-pShMKS1::ShMKS1 showed increased transcript levels of both ShMKS2 and ShMKS1 in trichomes but not in the leaves without trichomes (Fig. 5D). For comparison with transgenic Arabidopsis and tobacco plants expressing MKS2 under the control of the 35S promoter, we also generated plants carrying p35S::ShMKS2 (despite repeated attempts, we could not generate viable transgenic tomato plants expressing the p35S::ShMKS2-pRbcs::ShMKS1 construct).

The cultivated tomato lines expressing p35S::ShMKS2 showed lesions in their leaves (Fig. 6), accumulation of myristic acid, and a statistically significant decrease in the levels of C16:3 and C18:3 (Table IV), but they emitted no methylketones (data not shown). The six lines expressing only ShMKS2 and seven lines expressing both ShMKS1 and ShMKS2 in the trichomes were morphologically indistinguishable from wild-type cultivated tomato plants and they showed similar 5- to 7-fold increases in the content of individual methylketones in the trichomes compared with wild-type cultivated tomato trichomes, although only the increases measured in the lines containing...
the pShMK$S_2$:ShMK$S_2$ construct were statistically significant (Table V). However, the methylketone content in both the wild type and these transgenic cultivated tomato lines was negligible compared with the methylketone content found in trichomes of wild tomato (Table V). In addition, only pShMK$S_2$:ShMK$S_2$-pShMK$S_1$:ShMK$S_1$ plants showed significant increases in the concentrations of some of the fatty acids in their trichomes (Table VI).

**DISCUSSION**

**Trichome-Specific Coexpression of Both ShMK$S_1$ and ShMK$S_2$ Is Insufficient for Methylketone Production in Cultivated Tomato**

Methylketone production and accumulation in wild tomato (accession no. PI126449) renders it resistant to many cultivated tomato pests (Gonçalves et al., 1998). Introducing methylketone production into cultivated tomato is thus a desired goal for tomato breeders. However, previous tomato breeding efforts involving interspecific crosses showed that it was difficult to breed tomato lines with high levels of methylketone production in their trichomes (Hartman and St. Clair, 1998), consistent with early genetic analysis showing that the methylketone production trait in tomato was associated with multiple gene loci (Zamir et al., 1984). Recent genetic and molecular analyses of cultivated tomato × wild tomato hybrid progeny showed that that transcripts of several fatty acid biosynthesis genes were present at lower concentrations in the trichomes of cultivated tomato than in wild tomato, and that maximal levels of methylketones depended on the presence of wild tomato alleles at several fatty acid biosynthesis gene loci as well as at the MK$S_1$ and MK$S_2$ gene loci (Ben-Israel et al., 2009). Yet because the expression levels of ShMK$S_1$ and ShMK$S_2$ in wild tomato (accession no. PI126449) are respectively 800-fold and 300-fold higher than in the cultivated tomato and the differences in levels of transcripts of other fatty acid-related genes in the trichomes was only <8-fold between the two species, it was thought possible that the introduction of ShMK$S_1$ and ShMK$S_2$ alone might lead to increases in methylketone production in cultivated tomato plants.

Because ShMK$S_1$ and ShMK$S_2$ show trichome-specific expression in wild tomato (accession no. PI126449), the promoters of these two gene loci were amplified and fused in frame with their own ORF for trichome-specific expression in cultivated tomato. Transgenic cultivated tomato plants expressing ShMK$S_1$ and ShMK$S_2$ specifically in the trichomes showed a 6.5-fold increase in total methylketone concentrations in the trichomes (Table V). A similar result was obtained with transgenic lines expressing only ShMK$S_2$ in the trichome (Table V), suggesting that the rate of decarboxylation is not a limiting factor in tomato trichomes. However, because the concentration of methylketones in wild tomato trichomes is approximately 650-fold greater than in the trichomes of wild-type tomato plants, the transgenic cultivated tomato plants expressing ShMK$S_1$ and ShMK$S_2$ (or only ShMK$S_2$) in the trichomes still had methylketone concentrations in their trichomes that were 100-fold lower than those found in wild tomato trichomes (Table V). Thus, it appears that introducing only ShMK$S_1$ and ShMK$S_2$ into tomato for trichome-specific expression is not sufficient by itself to achieve a substantial increase in methylketone production. Successful engineering of tomato trichomal methylketone production might require increases in metabolic flux toward fatty acid biosynthetic pathway in trichomes, possibly by introducing the wild tomato fatty acid biosynthesis gene

### Table IV. Fatty acid compositions of transgenic and wild-type cultivated tomato seedlings (15 DAG)

Data are presented as the mean ± se. The se values were calculated from three biological measurements. An 8 DAG cultivated tomato plant has too little true leaf materials, so 15 DAG seedlings were analyzed. N.D., Not detected.

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>C14</th>
<th>C16</th>
<th>C16:3</th>
<th>C18</th>
<th>C18:2</th>
<th>C18:3</th>
<th>Total</th>
<th>µmol/g FLW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivated tomato</td>
<td>N.D.</td>
<td>2.28 ± 0.29</td>
<td>0.78 ± 0.02</td>
<td>0.16 ± 0.02</td>
<td>1.83 ± 0.19</td>
<td>6.34 ± 0.50</td>
<td>11.40 ± 0.90</td>
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</tr>
<tr>
<td>P35S:ShMK$S_2$</td>
<td>0.12 ± 0.01</td>
<td>2.42 ± 0.25</td>
<td>0.31 ± 0.01</td>
<td>0.20 ± 0.03</td>
<td>1.73 ± 0.33</td>
<td>4.13 ± 0.28</td>
<td>8.92 ± 0.87</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>2-Undecanone</th>
<th>2-Tridecanone</th>
<th>2-Pentadecanone</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivated tomato</td>
<td>0.0036 ± 0.0016</td>
<td>0.0724 ± 0.0342</td>
<td>0.0056 ± 0.0008</td>
<td>0.0816 ± 0.0335</td>
</tr>
<tr>
<td>pShMK$S_2$:ShMK$S_2$</td>
<td>0.0251 ± 0.0159</td>
<td>0.4949 ± 0.1508</td>
<td>0.0246 ± 0.0009</td>
<td>0.5445 ± 0.1647</td>
</tr>
<tr>
<td>pShMK$S_2$:ShMK$S_1$:pShMK$S_1$:ShMK$S_1$</td>
<td>0.0205 ± 0.0081</td>
<td>0.5044 ± 0.2047</td>
<td>0.0278 ± 0.0143</td>
<td>0.5527 ± 0.2272</td>
</tr>
<tr>
<td>Wild tomato</td>
<td>15.9049 ± 5.2889</td>
<td>36.2958 ± 11.1823</td>
<td>2.6446 ± 1.3428</td>
<td>54.8453 ± 17.7450</td>
</tr>
</tbody>
</table>

aP < 0.05. bP < 0.01.
Table VI. Fatty acid compositions of isolated tomato trichomes

Data are presented as the mean ± s.e. The s.e. values were calculated from three biological measurements. FTW, fresh trichome weight; N.D., not detected.

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>C12</th>
<th>C14</th>
<th>C16</th>
<th>C16-3</th>
<th>C18</th>
<th>C18-1</th>
<th>C18-2</th>
<th>C18-3</th>
<th>C20</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivated tomato</td>
<td>1.87 ± 1.24</td>
<td>1.50 ± 1.07</td>
<td>2.07 ± 2.07</td>
<td>0.58 ± 0.58</td>
<td>0.93 ± 0.93</td>
<td>0.44 ± 0.44</td>
<td>0.24 ± 0.24</td>
<td>0.03 ± 0.03</td>
<td>0.82 ± 0.82</td>
<td>3.13 ± 3.13</td>
</tr>
<tr>
<td>ShMKS1</td>
<td>1.61 ± 2.11</td>
<td>1.08 ± 0.67</td>
<td>0.67 ± 0.67</td>
<td>0.72 ± 0.72</td>
<td>0.66 ± 0.66</td>
<td>0.10 ± 0.10</td>
<td>0.01 ± 0.01</td>
<td>3.66 ± 3.66</td>
<td>1.37 ± 1.37</td>
<td>3.89 ± 3.89</td>
</tr>
<tr>
<td>N.D.</td>
<td>0.91 ± 0.28</td>
<td>3.25 ± 3.25</td>
<td>0.34 ± 0.34</td>
<td>1.22 ± 1.22</td>
<td>0.70 ± 0.70</td>
<td>0.03 ± 0.03</td>
<td>0.01 ± 0.01</td>
<td>3.70 ± 3.70</td>
<td>1.17 ± 1.17</td>
<td>3.34 ± 3.34</td>
</tr>
<tr>
<td>pShMKS2</td>
<td>2.02 ± 1.02</td>
<td>3.25 ± 3.25</td>
<td>0.35 ± 0.35</td>
<td>1.22 ± 1.22</td>
<td>0.70 ± 0.70</td>
<td>0.03 ± 0.03</td>
<td>0.01 ± 0.01</td>
<td>2.60 ± 2.60</td>
<td>0.94 ± 0.94</td>
<td>2.54 ± 2.54</td>
</tr>
<tr>
<td>pShMKS2::ShMKS1</td>
<td>1.93 ± 1.93</td>
<td>1.93 ± 1.93</td>
<td>1.93 ± 1.93</td>
<td>1.93 ± 1.93</td>
<td>1.93 ± 1.93</td>
<td>1.93 ± 1.93</td>
<td>1.93 ± 1.93</td>
<td>1.93 ± 1.93</td>
<td>1.93 ± 1.93</td>
<td>1.93 ± 1.93</td>
</tr>
<tr>
<td>Wild tomato PI126449</td>
<td>2.82 ± 2.82</td>
<td>2.11 ± 2.11</td>
<td>1.08 ± 1.08</td>
<td>0.72 ± 0.72</td>
<td>0.66 ± 0.66</td>
<td>0.10 ± 0.10</td>
<td>0.01 ± 0.01</td>
<td>3.66 ± 3.66</td>
<td>1.37 ± 1.37</td>
<td>3.89 ± 3.89</td>
</tr>
</tbody>
</table>

Methylketone Production in Transgenic Plants Expressing ShMKS2 under the Control of the General Promoter 35S, with or without Coexpression of ShMKS1

Coexpression of ShMKS1 and ShMKS2 in Arabidopsis and tobacco led to methylketone production in planta, indicating that methylketone production could be engineered in other plant species. In a 5-h period, transgenic tobacco plants expressing both ShMKS1 and ShMKS2 produced 12.93 μmol/g FLW methylketones, whereas the Arabidopsis counterparts produced only 1.83 μmol/g FLW (Table II).

The difference in rates of methylketone synthesis between the Arabidopsis and tobacco might be a result of the tobacco seedlings having a higher fatty acid flux rate. In tobacco, expression of ShMKS2 alone also led to the synthesis of methylketones at 2.82 μmol/g FLW/5 h, which was 80% lower than the rate of methylketone production by transgenic tobacco plants expressing both ShMKS1 and ShMKS2. On the other hand, transgenic Arabidopsis and tomato plants expressing only ShMKS2 under the control of the 35S promoter emitted no methylketones (Table II). The action of the thioesterase ShMKS2 on its β-ketoacyl-ACP substrates leads to the production of 3-keto acids, which are known to be unstable and to spontaneously decarboxylate to give the corresponding 2-methylketones (Matiasek et al., 2001). A previous study showed that expressing ShMKS2 in E. coli also leads to the appearance of methylketones (Yu et al., 2010). In addition, the spent medium of the bacteria accumulated 3-keto acids (Yu et al., 2010). These observations demonstrate that the action of the decarboxylase ShMKS1 is not absolutely required to produce methylketones. However, the expression of ShMKS1 in addition to ShMKS2 led to a 5-fold increase in methylketone production in transgenic tobacco, consistent with the results of the coexpression of the two genes in E. coli (Park et al., 2012).

The fate of the 3-keto acids produced by the action of ShMKS2 in planta but not converted to methylketones is not clear. We could not detect any 3-keto acids in Arabidopsis, tobacco, and tomato plants expressing ShMKS2. These plants, except tobacco, emitted no methylketones. Yet because Arabidopsis as well as tobacco plants expressing both ShMKS1 and ShMKS2 emit methylketone, a considerable amount of 3-keto acids must have been generated in plants expressing ShMKS2 alone. The 3-keto acids may have been catabolized by the plants at a faster rate than their spontaneous degradation rate, which could also explain the lower methylketone productivity of tobacco plants expressing only ShMKS2 than that of tobacco plants expressing both ShMKS1 and ShMKS2.

ShMKS2 Is Capable of Hydrolyzing Myristoyl-ACP in Addition to 3-Ketoacyl-ACP

Transgenic Arabidopsis and tobacco plants expressing ShMKS2 together with ShMKS1 produced similar
methylketones, and at similar ratios, as those of the wild tomato (accession no. PI126449; Supplemental Fig. S1B), with 2-tridecanone being the major methylketone in all cases. This suggests that the substrate chain-length selectivity of ShMKS2 is not changed when heterologously expressed in other plant species. Interestingly, production of myristic acid was observed in all three plant species expressing ShMKS2 under the 35S promoter (Tables I, III, and IV), indicating that ShMKS2 is capable of hydrolyzing myristoyl-ACP as well. An examination of the trichomes of wild tomato (accession no. PI126449) showed that low levels of myristic acid were found in these trichomes as well (Table IV). Thus, although ShMKS2 is mostly a β-ketoacyl-ACP thioesterase preferring the C14 β-ketoacyl-ACP substrate, it is also capable of hydrolyzing a fully reduced myristoyl-ACP although evidently at a lower rate.

Constitutive Expression of ShMKS2 in Plants Results in Phenotypes Similar to Those of Arabidopsis Mutants with Defects in de Novo Fatty Acids Biosynthesis

The morphological and developmental phenotypes observed for ShMKS2-expressing plants were quite similar to those of Arabidopsis mutants that are deficient in de novo fatty acid biosynthesis, such as the Arabidopsis acyl-ACP thioesterase mutant FATB, the enoyl-ACP reductase mutant mastic death1, and the β-ketoacyl-ACP synthase1 mutant (Mou et al., 2000; Bonaventure et al., 2003; Wu and Xue, 2010), all of which display growth retardation, variegated leaves, and reduced fertility. It is also interesting to note that the deleterious effects of the β-ketoacyl-ACP synthase1 mutant were alleviated when the plants matured (Wu and Xue, 2010), similar to our observations with older Arabidopsis and tobacco plants expressing ShMKS2 (Figs. 1, K–N, and 3, G–I). However, although the phenotypes observed are consistent with an interference with fatty acid biosynthesis as predicted based on the function of ShMKS2, which is a thioesterase utilizing the intermediates in fatty acid biosynthesis as substrates (Yu et al., 2010) and thus terminating the fatty acid biosynthesis prematurely, the actual measurements of fatty acid concentrations in the transgenic plants appear to be inconsistent with this hypothesis. Specifically, fatty acid concentrations in general increased in transgenic Arabidopsis plants, whereas they either did not decrease or decreased only slightly in transgenic tobacco and tomato plants expressing ShMKS2, and not all fatty acids were equally affected (Tables I, III, and IV). It is worth noting that expressing a short-chain thioesterase in the alga Phaeodactylum tricornutum also led to a simultaneous increase in total fatty acids and a decrease in growth rate (Radakovits et al., 2011).

Although interference with fatty acid biosynthesis remains a possible explanation for the lesions and the delayed growth (albeit without a clear, specific mechanism), there may be other alternative explanations. For example, the 3-ketoacids or their metabolites, including methylketones, might also lead to toxic effects on the plant, which could explain the persistent developmental problems seen in these plants, particularly transgenic tobacco plants expressing both ShMKS1 and ShMKS2 that made high levels of methylketones (Fig. 3, G–I). Thus, attempts to engineer high-level production of methylketones throughout the plant are likely to fail. To avoid the deleterious effects caused by interference with fatty acid biosynthesis or the production of toxic products, the expression of ShMKS1 and ShMKS2 must be directed to a small, self-contained structure such as trichomes or root nodules.

MATERIALS AND METHODS

Plasmid Construction

Plasmids for plant transformation were constructed by employing the pSAT vector system (Tzfira et al., 2005). The full-length coding sequences were end-fused in frame with EcoRI and Sall restriction sites for ShMKS1 and BglII and Sall sites for ShMKS2 by PCR. Next, these constructs were spliced into pSAT4A–35S to build p35S::ShMKS1 and p35S::ShMKS2. The ShMKS1 fragment was also transferred to pSAT6a-rbc to form pRbc::ShMKS1. The 35S promoter of p35S::ShMKS2 was exchanged for the promoter sequence of ShMKS2 by AgeI and BglII to build the pShMKS2-MKS2 construct. The pShMKS1-MKS1 construct was made by switching the RbcS promoter with the promoter sequence of ShMKS1 by AgeI and EcoRI. The pShMKS2::GFP construct was obtained by splicing the promoter sequence of ShMKS2 into AgeI and BanII restriction sites of pSAT6a-EFGP construct to replace the 35S promoter (Tzfira et al., 2005).

All of the pSAT4A expression cassettes were spliced into the I-SceI site and pSAT6a expression cassettes were spliced into the PL-PgiI site of the binary vector pPZF-RC2 (Tzfira et al., 2005). For p35S::ShMKS2-pRbc::ShMKS1 and pShMKS1::ShMKS1-pShMKS2::ShMKS2, two expression cassettes were spliced in the same binary vector.

Plant Materials and Generation of Transgenic Plants

Wild-type (Columbia 0) Arabidopsis thaliana or transgenic seeds were surface sterilized with 70% (v/v) ethanol for 10 min and rinsed with 100% (v/v) ethanol to remove water. The seeds were then left in culture tubes at 37°C to completely dry up the ethanol. The dry seeds were spread on half-strength MS (Sigma-Aldrich) phytohormone (Calgon Labs) plates containing 1% (v/v) Suc. After 3 d of stratification at 4°C, plates were left in a growth chamber that was adjusted to 14-h light/10-h dark at 22°C. The same growth room condition was used for all plants mentioned in this study, unless otherwise stated. Seedlings were harvested at 8 DAG for fatty acid analysis and the rest were transferred onto soil. Plants in soil were harvested again at 35 DAG.

Nicotiana tabacum (cv. SR1) seeds were surface sterilized with 40% (v/v) Clorox bleach for 15 min and rinsed with sterilized water, and then transferred (with some water) onto half-strength MS plates. Tobacco (N. tabacum) seedlings were transferred to soil at 15 DAG after volatiles had been collected.

Solanum lycopersicum MPl seeds were sterilized with 50% (v/v) bleach for 20 min and then with 70% (v/v) ethanol for 10 min. The seeds were then dried and spread onto half-strength MS plates.

For selection of transgenic plants, Arabidopsis, tobacco, and cultivated tomato plants were selected on half-strength MS plates containing 50 mg/L, 200 mg/L, and 200 mg/L kanamycin, respectively. ShMKS1 was amplified by oligonucleotide-pair 5'-GAATTCTAGGAAAGACATGTGCC-3' and 5'-GGGCTCGACTTTTATCTGATCGTCT-3' and ShMKS2 was
amplified using 5′-AGGATCTAGGTCCGAGGGTTCAGCAT-3′ and 5′-GTGACTTACAGGGTATGCATCCACGCCA-3′.

Trichome Isolation and Swabbed Leaves

Trichomes were isolated by gently shaking the leaves taken right out of liquid nitrogen in a 50 mL corning tube. After discarding the leaves, the frozen trichomes were washed off the tube by the RNA extraction buffer and ready for isolation. Trichomes on leaves were removed by using cotton swabs to brush the leaf surface several times.

RT-PCR Analysis of Transgenic Plants

RNA was extracted from the leaves of the wild-type and transgenic plants using an E.Z.N.A. Plant RNA Kit (Omega Bio-tek). During the RNA isolation, the DNA was removed by the On-Column DNase I Digestion Set (Sigma-Alrich) and the purified RNA was then reverse transcribed by the High Capacity cDNA Reverse Transcription Kit (Life Technologies). PCR reactions were performed by GoTaq Green Master Mix (Promega).

Plant Imaging

Plant phenotype images were taken through a Leica MZ66 anatomy microscope or directly by a Nikon Coolpix 4500 camera. The fluorescence anatomy microscope was a Leica MZFLIII, with all factory-equipped devices and default settings for bright-field and green-fluorescence imaging. Confocal microscopy was detailed in Yu et al. (2010), with images taken through a ×20 objective lens.

Fatty Acid and Methylketone Analysis by GC-MS

Fatty acid analysis followed the procedure described by Browse et al. (1986) with a small modification. Briefly, approximately 100 mg of fresh seedlings or mature plant leaf material were weighed and submersed in 1 mL 1N methanol-HCL anhydrous solution (with 0.1 mM pentadecanoic acid as an internal standard) for 2 h at 60°C for fatty acid extraction and derivatization. After the reactions were cooled to room temperature, 1 mL of 0.9% NaCl was added and then the mixture was extracted by 300 μL hexane. The hexane layer was used directly for GC-MS analysis. The hexane extract (3 μL) was injected in splitless mode into a Shimadzu GCMS-QP5000 system equipped with an EC-WAX column (thickness, 0.25 μm; diameter, 0.32 mm; length, 30 m). The initial oven temperature of the gas chromatograph was 50°C, which was then immediately ramped to 250°C at 5°C/min and held at 250°C for 1 min. The injector and detector were set at 250°C and 280°C, respectively. The quantities of each fatty acid were calculated by comparing the peak areas with that of the pentadecanoic acid.

Methylketones were analyzed on the same GC-MS machine, but the oven temperature was held at 50°C for 2 min and then ramped at 10°C/min to 190°C, followed by 5°C/min to 220°C, and finally 10°C/min to 275°C. The temperature was held at 275°C for 3 min. Helium was used as the carrier gas and the flow rate was 1.4 mL/min for both methods.

The identities of fatty acids, methylketones, and the medium-chain 2-alcohols were determined by comparing their retention times and mass spectra with those of authentic chemical standards. The identity of 2-tridecanol was inferred from comparing its mass spectrum and retention time with that of 2-dodecanol and 2-tetradecan.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure S1. Chromatography of wild-type Arabidopsis and wild tomato.

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