Improved herbivore resistance in cultivated tomato with the sesquiterpene biosynthetic pathway from a wild relative

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Tomato breeding has been tremendously efficient in increasing fruit quality and quantity but did not focus on improving herbivore resistance. The biosynthetic pathway for the production of 7-epizingiberene in a wild tomato was introduced into a cultivated greenhouse variety with the aim to obtain herbivore resistance. 7-Epizingiberene is a specific sesquiterpene with toxic and repellent properties that is produced and stored in glandular trichomes. We identified 7-epizingiberene synthase (ShZIS) that belongs to a new class of sesquiterpene synthases, exclusively using Z-Z-farnesyl-diphosphate (zFPP) in plastids, probably arisen through neo-functionalization of a common ancestor. Expression of the ShZIS and zFPP synthase in the glandular trichomes of cultivated tomato resulted in the production of 7-epizingiberene. These tomatoes gained resistance to several herbivores that are pests of tomato. Hence, introduction of this sesquiterpene biosynthetic pathway into cultivated tomatoes resulted in improved herbivore resistance.

Herbivorous insects pose serious problems in agricultural production areas. On commercial tomato, whiteflies, spider mites, and aphids are major pests, not only by the feeding damage they cause, but also because they transmit devastating viruses that can cause losses up to 100% (1). Production of tomato, a crop with considerable economic importance, reached 145,751,507 tons worldwide in 2010, representing a value of 53.3 billion dollars (www.fao.org). In general, wild tomatoes are far less attractive to pests than cultivated tomatoes due to an elevated or qualitatively different production of an array of defense compounds such as alkaloids, phenolic compounds, and terpenes.

Toxic and repellent compounds are mostly produced in glandular trichomes, autonomous epidermal protrusions specialized in efficient production, storage, and release of defense compounds (2). These glands have long been regarded as economically important and challenging targets for bioengineering (2). Confining metabolic changes to trichomes ensures that the plant metabolism and crop yield remain unaffected.

Terpenes constitute the largest and most diverse class of plant-produced secondary compounds that maintain a variety of biological functions and applications, including anticancer (taxol) and antimarial (artemisin) drugs, in flavor and fragrance industry, and for crop improvement through enhanced pest resistance. Wild tomato sesquiterpenes and derivatives have been implicated in defense against herbivores (3–5). Sesquiterpenes are C_{15}-terpenes predominantly derived from precursors of the cytosolic mevalonate pathway where the C_{5}-isoprene units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are assembled into C_{15} E-E-farnesyl diphosphate (FPP) by FPP synthase (FPS) and converted by specific sesquiterpene synthases. However, it was recently shown that some wild tomato species contain a sesquiterpene pathway that is confined to the chloroplast (6). In Solanum habrochaites LA1777 an atypical sesquiterpene synthase produces the sesquiterpenes santalene and bergamotene from Z-Z-farnesyl diphosphate (Z,Z-FPP) that, in turn, is produced in the plastids from IPP and DMAPP by a short chain cis-prenyltransferase (cZPS) (6). Recently an additional S. habrochaites sesquiterpene synthase has been identified, along with two new, cis-substrate (neryl diphosphate) using, monoterpene synthases (7). On a sequence level, this sesquiterpene synthase resembles plastidial diterpene synthases more than cytosolic sesquiterpene synthases. In cultivated tomato, the plastidial sesquiterpene synthase pathway appears to be absent, although another cis-prenyltransferase, i.e., neryl-diphosphate synthase, is responsible for producing the precursor of several C_{10}-monoterpenes (8). Cis-prenyltransferases have evolved independently from trans-prenyltransferases (9). Zingiberene is a sesquiterpene important in plant defense against an array of herbivores due to its toxicity and repellence (10–14) and is found in a variety of plant species including basil, turmeric, cardamom, and sorghum. Recently, we found that tomato contains 7-epizingiberene, a stereoisomer of α-zingiberene (15). Importantly, purified 7-epizingiberene, when applied to the headspace of susceptible cultivated tomato, repelled whiteflies, whereas α-zingiberene isolated from ginger did not (15). Although several zingiberene synthases have been identified (16–18), including one from S. habrochaites (7), we have now unambiguously identified and characterized 7-epizingiberene synthase from S. habrochaites and modified a cultivated tomato to produce 7-epizingiberene. Moreover, plants with improved herbivore resistance were obtained by traditional breeding and these may contribute to reduced pesticide use.

Results

Role for 7-Epizingiberene in Defense Against an Agricultural Pest. To confirm the biological relevance of 7-epizingiberene in defense against whiteflies, an interspecific cross between Solanum lycopersicum cv Moneymaker and the wild tomato S. habrochaites PI127826 was created. PI127826 contains high levels of 7-epi-
zingiberene (15), which the cultivated tomato lacks. The segregating F2 plants, resulting from F1 selfings, produced 7-epi-zingiberene in a range of concentrations up to PI127826 levels (Fig. S1A). Six of the 7-epi-zingiberene containing F2 plants were selected for a terpene-profile analysis. Leaf-surface terpenes were analyzed by GC-MS (Fig. L4 and Fig. S1B). F2-2 contains zingiberene levels equal to PI127826 (10.7 ± 2.0, 14.6 ± 3.4 ng·mg⁻¹ FW, respectively). However, it also accumulated monoterpenes and/or other sesquiterpenes at high concentrations (Fig. S1B). F2-7, F2-71, and F2-141 produced medium levels of zingiberene (Fig. L4) and terpene levels more comparable to the cultivated plant, both in quantity and composition (Fig. S1B). Therefore, these plants were selected for whitefly bioassays. F2-141 contained only 8% 7-epi-zingiberene compared with the wild tomato and no significant effect on adult mortality was recorded (P = 0.4), although the number of eggs deposited was lower. After 5 d, 67% of the whiteflies were dead on PI127826 and F2-7 (Fig. 1B) whereas mortality on F2-71, containing very few terpenes other than zingiberene, reached 44%. Overall, the data show a correlation between the concentration of 7-epi-zingiberene on the leaf surface and the performance of whiteflies (Fig. S2). On all zingiberene-producing F2s, a 40–74% reduction in egg deposition was found compared with the cultivated parent (Fig. 1C), showing that tomato zingiberene is toxic to the insect pest *Bemisia tabaci* in lower doses than produced by the wild tomato plant.

**Identification of 7-Epizingerene Synthase.** To identify the biosynthetic pathway for 7-epi-zingiberene production in tomato, we determined which terpenes in PI127826 are derived from the cytosolic (MVA; mevalonate) and which from the plastidial (MEP: 2-C-methyl-D-erythritol 4-phosphate) pathway. To this end, we grew plants in hydroponics with the MEP-pathway inhibitor fosmidomycin. In treated plants, the levels of monoterpenes, which are also derived from precursors of the MEP pathway, were reduced by 80% on average (Table 1). Whereas cytosolic sesquiterpenes such as germacrenes were slightly elevated in fosmidomycin-treated plants, 7-epi-zingiberene and its derivative R-curcumene decreased by 86% and 99.5%, respectively. Also levels of β-sesquiphellandrene were reduced, indicating that these three sesquiterpenes have a plastidial origin.

Salleau et al. (6) first provided evidence for a plastid localized sesquiterpene synthase; santalene-bergamotene synthase (SBS) from *L. albus*. Based on this sequence we cloned a 778 amino acid terpene synthase from PI127826 with 96% similarity to SBS (Fig. S3A). By 86% and 99.5%, respectively. Also levels of β-sesquiphellandrene were reduced, indicating that these three sesquiterpenes have a plastidial origin. In treated plants, the levels of monoterpenes, which are also derived from precursors of the MEP pathway, were reduced by 80% on average (Table 1). Whereas cytosolic sesquiterpenes such as germacrenes were slightly elevated in fosmidomycin-treated plants, 7-epi-zingiberene and its derivative R-curcumene decreased by 86% and 99.5%, respectively. Also levels of β-sesquiphellandrene were reduced, indicating that these three sesquiterpenes have a plastidial origin.

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![Fig. 1. Zingiberene levels influence whitefly performance on F2 plants of an interspecific cross between *S. lycopersicum* cv Moneymaker and *S. habrochaites* PI127826 and both parents. (A) Leaf surface 7-epi-zingiberene (ng·mg⁻¹ FW leaf) (n = 5–8). (B) Whitefly mortality (% of total). (C) Whitefly fecundity displayed as the total number of eggs per leaf (n = 5–11). Bars represent means ± SE, different letters signify statistical differences (P < 0.05).](image-url)

**Table 1. Terpene concentrations (ng·mg⁻¹ leaf FW) in leaf wash of *S. habrochaites* PI127826 cuttings grown for 5 wk in hydroculture amended with 10 μM fosmidomycin**

<table>
<thead>
<tr>
<th>Terpene</th>
<th>Control</th>
<th>Treated</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Phellandrene</td>
<td>0.11 ± 0.01</td>
<td>0.04 ± 0.00</td>
<td>0.001</td>
</tr>
<tr>
<td>α-Terpine</td>
<td>0.10 ± 0.01</td>
<td>nd</td>
<td>0.002</td>
</tr>
<tr>
<td>α-limonene</td>
<td>0.28 ± 0.03</td>
<td>0.09 ± 0.01</td>
<td>0.002</td>
</tr>
<tr>
<td>γ-Terpine</td>
<td>0.04 ± 0.01</td>
<td>nd</td>
<td>0.005</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>0.06 ± 0.01</td>
<td>nd</td>
<td>0.007</td>
</tr>
<tr>
<td>γ-Elemene</td>
<td>0.02 ± 0.00</td>
<td>0.12 ± 0.06</td>
<td>ns</td>
</tr>
<tr>
<td>Sesquibainene</td>
<td>0.05 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>ns</td>
</tr>
<tr>
<td>R-curcumene</td>
<td>0.30 ± 0.08</td>
<td>0.00 ± 0.00</td>
<td>0.017</td>
</tr>
<tr>
<td>7-Epizingerene</td>
<td>7.64 ± 1.82</td>
<td>1.05 ± 0.33</td>
<td>0.006</td>
</tr>
<tr>
<td>Germacrene A</td>
<td>0.04 ± 0.01</td>
<td>0.08 ± 0.03</td>
<td>ns</td>
</tr>
<tr>
<td>β-Sesquiphellandrene</td>
<td>0.23 ± 0.04</td>
<td>0.03 ± 0.03</td>
<td>0.015</td>
</tr>
<tr>
<td>α-Cadinene</td>
<td>0.05 ± 0.00</td>
<td>0.18 ± 0.11</td>
<td>ns</td>
</tr>
<tr>
<td>Selina-3,7 (11)-diene</td>
<td>0.12 ± 0.02</td>
<td>0.25 ± 0.16</td>
<td>ns</td>
</tr>
<tr>
<td>Germacrene B</td>
<td>1.70 ± 0.34</td>
<td>4.77 ± 3.18</td>
<td>ns</td>
</tr>
</tbody>
</table>

Data are means ± SE, n = 3. nd, not detected; ns, not significant.
from four other zingiberene-producing *S. habrochaites* accessions (LA0094, LA1978, LA2167, and LA2650). Comparative analyses revealed 10 SNPs of which 7 led to amino acid changes (Table S1).

**Functional Characterization of ShZIS.** Recombinant ShZIS enzyme exclusively used Z,Z-FPP and was unable to synthesize detectable products from any other terpene precursor offered, including the stereoisomer *E,E*-FPP (Fig. 2A). Using Z,Z-FPP, the enzyme made zingiberene (98.6%) and trace amounts of β-sesquiphellandrene (0.8%) and curcumene (0.6%), the dehydrogenated derivative of zingiberene. Recombinant zingiberene synthase from lemon basil (ObZIS) (16, 17) was used for comparison with ShZIS (Fig. 2B). ObZIS produced mainly zingiberene from *E,E*-FPP whereas Z,Z-FPP was converted mostly into α-bergamotene. Moreover, ObZIS was able to convert both C10 precursors GPP and NPP into monoterpenes, although at lower efficiency.

Enantioselective gas chromatography on a β-cyclodextrin coated column allowed identification of the different zingiberene stereoisomers from tomato and ginger (Fig. 3A), previously shown to contain 7-epizingiberene and α-zingiberene, respectively (15). We established that recombinant tomato ShZIS indeed produced 7-epizingiberene from Z,Z-FPP, whereas ObZIS is a *bona fide* α-zingiberene synthase that uses *E,E*-FPP (Fig. 3A). The kinetic constants for recombinant ShZIS (±84 kDa) indicated a substrate affinity and turnover rate comparable to other plant sesquiterpene synthases e.g., the maize (*E*)-β-caryophyllene synthase and tobacco 5-epiaristolochene synthase (21, 22), with a *Kₘ* value of 7.12 ± 2.68 μM Z,Z-FPP and *Kₐ* of 0.29 ± 0.10 s⁻¹.

Engineering of *zFPS* and ShZIS in Cultivated Tomato and Herbivore Responses. The F2 plants that produced 7-epizingiberene (Fig. 3B) contained both the *zFPS* and the ShZIS genes that are specifically expressed in trichomes (Fig. S4A). *S. habrochaites* PI127826 treated with jasmonic acid (JA), a plant hormone involved in response to herbivores, accumulated higher levels of 7-epizingiberene compared with mock-treated plants (Fig. S4B).

The function of ShZIS was assessed further by stable expression of *zFPS* and *ShZIS* genes in *S. lycopersicum* cv Moneymaker. The *zFPS* cDNA was expressed under control of the *S. habrochaites* Methyl Ketone Synthase (MKS1) (24) promoter. Transgenic tomato plants containing the uidA reporter (GUS) driven by the MKS1 or the MTS1 promoter revealed that expression under these promoters was specific for type VI glandular trichomes (Fig. S4 C and D). The terpene profiles of leaf material from seven independent primary *zFPS/ShZIS*-transformants were analyzed by GC-MS and compared with those of plants expressing *zFPS* alone (Fig. 4A). The transgenic lines containing both genes accumulated 7-epizingiberene (Fig. 4B), proving that 7-epizingiberene synthesis is orchestrated by ShZIS converting Z,Z-FPP in the plastid of tomato trichomes.

![Fig. 2. Enzymatic activity of recombinant ZIS enzymes. GC-MS chromatograms of SPME sampling of *S. habrochaites* PI127826 (A) and ObZIS from *O. basilicum* (AY693646) (B) expressed in *E. coli*, assayed with sesquiterpene precursor *E,E*-FPP (FPP) or Z,Z-FPP (ZFP) and measured by SPME. Peaks: 1: *E,E*-zingiberene 2: *E,E*-sesquiphellandrene, 3: 7-episesquithujene, 4: sesquithujene, 5: (Z)-α-bergamotene, 6: (E)-α-bergamotene, 7: (E)-γ-farnesene, 8: α-zingiberene 9: α-farnesene 10: β-bisabolene 11: (E)-nerolidol, 12: β-acoradiene, 13: (Z)-β-bisabolene, 14: (E)-γ-bisabolene. The detector response for terpene-specific ion 93 is shown.](image-url)
Coexpression of zFs and ShZis in tomato trichomes did not reduce levels of monoterpenes ($P = 0.20$).

Transgenic line 2 contained the highest levels of 7-epizingiberene and was used for bioassays with whiteflies and spider mites (the generalist Tetranychus urticae and a specialist of solanaceous plants, Tetranychus evansi). The leaf washes of line 2 contained only around 1.5% of the 7-epizingiberene in S. habrochaites PI127826. Whitefly mortality and total number of eggs deposited were not significantly affected. However, on transgenic plant 2, significantly more eggs were deposited on the abaxial leaf surface (87.0% ± 2.4) compared with an untransformed plant ($P = 0.01$) and the lines expressing zFPS alone ($P = 0.04$) (Fig. 4C). Whereas wild tomato has type IV and VI glandular trichomes on both sides of the leaf, cultivated tomato lacks type IV and has fewer type VI trichomes on the abaxial side (Fig. S5). Therefore, in cultivated tomato 7-epizingiberene will be produced mostly on the adaxial leaf surface in type VI trichomes, which inversely correlates to the distribution of whitefly eggs (Fig. S5E). The production of 7-epizingiberene had no repellent effect on spider mites in a choice assay ($P = 0.55$), but had a strong effect on their fecundity. Mites placed on leaf disks of transgenic line 2 exhibited a 40% higher mortality and, after 4 d, an 81% reduction in the number of eggs was recorded in the case of T. urticae (Fig. 4D). The fecundity of the invasive JA-response suppressing mite T. evansi was reduced by 54% on line 2 (Fig. S6). Subsequently, we tried to mimic the situation in a production greenhouse by infesting intact tomato plants with three female T. urticae mites and then monitored the population growth. The presence of 7-epizingiberene in the transgenic plants affected the population growth rates leading to significantly lower numbers ($P < 0.001$) of mites on the transgenic plants compared with control plants after 45 d (Fig. 4E). Moreover, the fecundity of spider mites, which had been allowed to adapt for two generations either on control or transgenic leaves, was still significantly lower on 7-epizingiberene producing transgenic plants independent from the selection regime (Fig. S7).

7-Epizingiberene also influenced the performance of different insect orders. The development of larvae of the tobacco hornworm (Manduca sexta; Lepidoptera) placed on 7-epizingiberene producing F2 plants (F2-100/21; Fig. S1B) was significantly ($P < 0.0001$) retarded compared with that on F2 plants without 7-epizingiberene (F2-200/2; Fig. S4A). Also, the Colorado Potato Beetle (Leptinotarsa decemlineata; Coleoptera) preferred a diet without 7-epizingiberene in a choice assay (Fig. S8B; $P < 0.01$).

**Discussion**

Wild tomato species possess a large genetic diversity and genes for the production of defense-related metabolites (25, 26). Many of these genes were presumably lost during cultivation of tomato leaving them hampered in their defenses against pests such as whiteflies and spider mites. Here we report that a naturally optimized biosynthetic pathway in the wild-tomato germplasm, responsible for the plant’s extremely high production of a specific sesquiterpene, can make susceptible cultivated tomatoes more resistant.
We identified ShZIS, a trichome-specific sesquiterpene synthase, to produce the 7-epi-stereoisofrom of zingiberene in wild tomato PI127826. The protein has high sequence homology with the recently discovered ShSBS plastidial sesquiterpene synthase from wild tomato LA1777 (6). In a recent survey several terpene synthases of the TPS-e/f family from S. habrochaites were identified to produce α-pinene, limonene or zingiberene (7). PI127826 ShZIS is identical to ShZIS from LA2167 recently identified (7) (GenBank accession no. J9990661), although we found a few SNPs in that accession (Table S1). We can conclude that a relatively high number of SNPs is present in 7-epi-zingiberene synthases of different S. habrochaites accessions (see also Table S1).

The presence of an N-terminal targeting peptide nearly identical to ShSBS (Fig. S34), and the fact that ShZIS exclusively accepts plastidial Z,Z-FPP (6) as a substrate (Fig. 2 and Table 1), indicates plastidial localization. Other zingiberene synthases from basil, sorghum, and grape, have promiscuous activity and convert cytosolic E,E-FPP into a variety of sesquiterpenes (17, 18, 27) (Fig. 2B). Tomato appears to possess a set of atypical terpene synthases, closely related to ancestral TPS synthases (Fig. S3B) that have so far not been found in other sequenced species (27).

PI127826 produces 7-epi-zingiberene in large amounts and appears to invest in a high and constant rate of production. Producing such extreme sesquiterpene levels could, perhaps, be facilitated by a plastidial biosynthetic pathway. Metabolic engineering of a sesquiterpene pathway to the chloroplast resulted in highly elevated sesquiterpene levels in tobacco (28). In S. habrochaites LA1777, the terpenoid profile is dominated by sesquiterpenes of plastidial origin (29) and it is likely that even more plastidial sesquiterpene synthases will be identified.

The presence of zingiberene could be assigned to a single major locus (30). We now have provided evidence that this locus must contain ShZIS. In LA1777, ShSBS and zFPP are located on the top of chromosome 8, in very close proximity to each other (6). We presume that ShZIS is allelic to ShSBS and linked to zFPP as well. Based on geographic distribution and evolutionary divergence, Gonzales-Vigil et al. (7) suggested that ShZIS originated before ShSBS. Because mutagenesis of only three amino acids in the active site of a predominantly zingiberene synthetizing enzyme resulted in an enzyme producing predominantly (E)-α-bergamo- terone, it seems likely that (E)-α-bisabolone (18) mutations could lead to divergence of ZIS and SBS genes in tomato (7). Probably the neofunctionalization of ShZIS and ShSBS enzymes occurred after a duplication event of an ent-kaurene synthase-like gene in the genome of S. habrochaites. Interestingly, SBS and ZIS genes share high homology (88–89%) to the S. pennellii and cultivated tomato monoterpane synthases, PHS (TPS20), on top of chromosome 8 (8, 20), which make α- and β-phellandrene, respectively.

The toxic effect of trichome exudates of the wild PI126826 tomato, attributed to the presence of zingiberene, has been associated with resistance against a variety of herbivores (10–13). The tomato stereoisomeric, 7-epi-zingiberene, appears to be a defensive compound in several ways. First, the constitutive presence of high amounts of 7-epi-zingiberene in the plant’s headspace probably acts as a semiochemical. Even though whitely antennae detect both zingiberene stereoisomers, repellence is specific for 7-epi-zingiberene (15). This repellence appears to be innate, but when given no alternative whitelyes will settle on a tomato emitting 7-epi-zingiberene and die (15). Second, 7-epi-zingiberene increases tomato-whitely fitness in a dose-dependent manner (Fig. S2).

We found a severe reduction in fecundity of two major tomato pests, whitelyes and spider mites (Figs. 1C and 4D and E). Survival rate and reproductive success of whitelyes on PI127826 and 7-epi-zingiberene producing F2s was severely compromised (Fig. 1). Even though the levels of 7-epi-zingiberene on the leaf surface of F2-7 and F2-71 were lower than in the wild tomato, exposure resulted in a comparable reduction of phytochemical level (Fig. 1). This will most likely also lead to reduced spread of Begomoviruses (26). When 7-epi-zingiberene levels were below a certain threshold, as was the case for F2-141 and transgenic line 2, a direct toxicity effect was not detected. However, the number of eggs was reduced (Fig. 1C) and with most glandular trichomes on the adaxial surface (Fig. S5), whitelyes appeared to respond by ovipositing away from the source of 7-epi-zingiberene production (Fig. 4C) as they do for trichome-produced acylsugars (31). Also herbivores belonging to different orders were severely affected by 7-epi-zingiberene. Neonate M. sexta larvae consume trichomes as a first meal (32), making them particularly sensitive to trichome-produced toxins as reflected in a severe reduction of body mass (Fig. S8A). The Colorado potato beetle was affected in its choice behavior by the presence of 7-epi-zingiberene (Fig. S8B).

The F2 plants might contain other traits influencing pest resistance, which we cannot completely exclude. For this reason we created transgenic lines identical to cultivated tomato in every sense, but for the production of 7-epi-zingiberene from their type VI glandular trichomes. In these lines the effect on spider mite fecundity can be attributed to the presence of 7-epi-zingiberene solely. 7-Epi-zingiberene production increased even further in response to JA treatment (Fig. S4B), indicating involvement in induced defenses following herbivory. The invasive red spider mite, T. evansi, a specialist on tomato, was recently found to suppress JA-activated defense responses (33), and is beginning to be a serious threat in agriculture. T. evansi appeared to perform slightly better than T. urticae on the transgenic line. However, also for this specialist, reproduction was severely compromised on the wild tomato and, more importantly, on the transgenic line (Fig. S6). The effect of 7-epi-zingiberene on mite survival and fecundity will affect population development on greenhouse tomatoes as exemplified by the multigenerational experiments (Fig. 4E and Fig. S7).

One trait influencing herbivore resistance is the type and density of the glandular trichomes that produce, store and emit 7-epi-zingiberene. Zingiberene levels had been associated to type IV and VI trichome densities in an F2-population (10). On cultivated tomato type IV trichomes are far outnumbered by type VI (refs. 10, 11, 34, and 35; Fig. S5) and the promoters used for expression of zFPP and ShZIS are active in type VI (Fig. S4), indicating they are the primary source of 7-epi-zingiberene produced (Fig. 4A and B). Therefore, we cannot exclude a contribution of other trichome types to the zingiberene production. Sole expression of zFPP and ShZIS is sufficient to allow S. lycopersicum to make repellent and toxic 7-epi-zingiberene, although concentrations are likely restricted by the number of trichomes (10, 11). Also, the promoters used in this study, chosen for their trichome specificity, are probably not as strong as the endogenous S. habrochaites promoters. We know that SIMTS1 is not highly expressed (24) and the ShMKS1 promoter is expressed out of context in S. lycopersicum, possibly influencing expression levels. However, because expression is restricted to glandular trichomes, transgenic lines developed normally and no effect on flowering, fruit and seed set was observed. Additionally, whereas it would be conceivable that expression of zFPP and ShZIS in the plastid would lead to precursor competition, we did not find lower levels of monoterpenes in the transgenic line.

In summary, tomato glandular trichomes can be used as bio-chemical factories for the production of toxic and repellent defense compounds. We exploited the fact that a wild tomato species contains an atypical terpene pathway, confined to the chloroplast, allowing these plants to produce exceptionally high concentrations of 7-epi-zingiberene. Trichomes emerge early in tomato ontogeny and are found all over the plant, except on ripe fruits, making them ideal for metabolic engineering without affecting fruit flavor and yield. We have shown that, by the addition of two enzymes in the terpenoid-biosynthetic pathway, it is feasible to alter insect-choice behavior and improve cultivated...
tomato defense, both as proof-of-concept in transgenic lines, as well as via non-GM techniques.

**Materials and Methods**

Detailed information is described in SI Materials and Methods.

**Plant Material.** Solanum habrochaites PI127826 was obtained from Keygene N.V. A cross between *S. lycopersicum* cv Moneymaker and PI127826 was made and an F2 population was obtained through selfing. Plants were grown in a controlled greenhouse compartment with 16 h light. For the inhibitor study cuttings were placed in a hydroponics system amended with 10 μM fosmidomycin.

**Expression Analyses.** Tomato trichomes were shaken from stems frozen in liquid nitrogen. RNA was isolated from trichomes for expression analyses using real-time quantitative PCR.

**GC-MS Analyses.** Total tissue terpenes were analyzed by Solid Phase Micro Extraction (SPME) followed by desorption into the injector port of the GC-Chromatograph Mass Spectrometer (GC-MS). For leaf surface terpenes, leaves were cut into terpene from trichome hexane. Leaf washes were analyzed by injection into the GC-MS injector port. Compounds were separated on a DB-5 column with helium as a carrier gas. Mass spectra were collected with a Time-of-Flight MS. Zingiberene stereoisomers were separated on an Astec CHIRALDEXTM B-DM column.

**Enzyme Characterisation.** ShZIS was cloned into the pGEX-KG expression vector and transformed into Escherichia coli C41. After induction activity assays were performed in the bacterial lysate by addition of various terpene precursors. Products were sampled by SPME and analyzed by GC-MS. For kinetic analyses the recombinant fusion protein was purified using glutathione affinity binding. Purified protein was assayed with different Z,Z-FPP concentrations to determine *Km* and *Kcat* values.

**In Plant Engineering.** Trichome specific expression of the Methyl Ketone Synthase (MK51) promoter and the Monoterpane Synthase (MTS1) promoter was confirmed by expression of the uidA reporter (GUS). Next, the MK51 promoter was used to drive ZFPS and the MTS1 promoter was cloned in front of ShZIS. Both constructs were transformed to *S. lycopersicum* cv Money-maker. The presence of 7-epizingiberene was analyzed by GC-MS in several independent transformants.

**Bioassays.** Performance of the Hemipteran *B. tabaci* (biotype Q) was assessed on F2 plants and transgenic lines producing 7-epizingiberene. Performance of spider mites (generalist *T. urticae* and specialist *T. evansi*; suborder Acanthosomatidae) was assessed in choice or no-choice assays on leaf disks and intact (transgenic) plants. The effect of 7-epizingiberene on the development of the Lepidopteran *M. sexta* and preference-behavior of the Coleopteran *L. decemlineata* was ascertained on F2 plants.

**Statistics.** Statistical analyses was performed by ANOVA followed by a post hoc test for comparison of individual means (SPSS Inc. 2010). When necessary values were log-transformed before analyses.

**Acknowledgments.** We thank Prof. Eran Pichersky for the basil zingiberene synthase and, with Yoko Iijima, for the MK51 promoter sequence; SUPELCO (www.sigmaaldrich.com) for help with development of the enantiomer GC-MS program; Ludek Tikovsky, Harold Lemereis, and Thijs Hendrix for plant care; the Max Planck Institute for Chemical Ecology (Jena, Germany) for the *M. sexta* eggs; and Nicole van Dam (Radboud University Nijmegen) for CPB eggs.

To separate zingiberene stereo-isomers, the Astec CHIR-ALDEX B-DM column (30 m × 0.25 mm × 0.12 μm; Supelco) was selected. The program was set to 115 °C for 3 min, increased to 140 °C by 4 °C·min⁻¹ where it was kept for 1 min, after which the temperature increased (2 °C·min⁻¹) to 166 °C where it was kept for 5 min before increase (40 °C·min⁻¹) to 220 °C. The injector temperature was kept at 220 °C, and column flow was kept at 1 μL·min⁻¹.

Enzyme Characterization. The candidate-sesquiterpene synthase from *S. habrochaites* PI127826 was amplified from trichome-enriched cDNA. After sequence verification the full-length cDNA was ligated into the pGEX-KG expression vector (2). The lemon-basil zingiberene synthase (3) was kindly provided by E. Pichersky (University of Michigan, MI). Constructs were transformed to C41 (DE3) electro competent *Escherichia coli* cells (4). An overnight culture was inoculated in Terrific Broth containing 100 μg·mL⁻¹ ampicillin. The culture was grown to OD₆₀₀ 0.5–0.6 at 37 °C, after which it was placed at 4 °C for 30 min. Expression was induced with 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) (Roche). After incubation (200 rpm, 16 °C, 16 h) cells were harvested (1,614 × g, 4 °C, 15 min). The pellet was resuspended in 2 mL assay buffer [AB; 25 mM Hepes (pH 7.2), 10 mM MgCl₂, 10% (vol/vol) glycerol] with lysozyme (1 mg·mL⁻¹) and Protease Inhibitor Mixture Complete (PICC, Roche). Cells were incubated on ice for 30 min and sonicated. Lysates were centrifuged (12,000 × g, 4 °C, 25 min) and stored at −80 °C. Activity assays were performed with 50 μL of bacterial lysate in 20-mL glass vials in a total volume of 500 μL AB supplemented with 5 mM DTT and either 2 μM Z,Z-FPP (2Z-6Z-farnesyl diphasphate), E,E-FPP (E-E-farnesyl diphasphate) GPP (geranyl diphasphate) NDP (neryl diphasphate) or GGPP (geranylgeranyl diphasphate) (Echelon Biosciences). Vials were closed with a Teflon-lined crimp cap and incubated (180 rpm, 30 °C, 1 h), after which headspace products were sampled by SPME.

To purify the GST-ZIS fusion protein, 250 mL of bacterial culture was grown and induced as described above. Cells were resuspended in 15 mL of extraction buffer (20 mM Tris·HCl, pH 7.5/100 mM NaCl/5 mM EDTA/1 mM EGTA/5 mM DTT) supplemented with 0.05% Tween 20 and 1× PICC. The recombinant protein was purified using glutathione Sepharose 4B resin (GE Healthcare, www.gelifesciences.com).

For kinetic analyses, 10 ng of purified recombinant protein was incubated with Z,Z-FPP (1–10 μM), in 500 μL of AB supplemented with 5 mM DTT. The mixtures were overlaid with 500 μL of hexane and incubated for 1 h at 30 °C. The reaction was stopped by vigorous mixing and centrifugation. The organic phase was collected followed by a second extraction with 500 μL of hexane. The combined extracts were dried with Na₂SO₄ injected on the GC-MS. Three replicates were performed for each Z,Z-FPP concentration. The *Kᵅ* and *Kₐₓ* values were determined from Lineweaver–Burk plots using Kaleidagraph software (Synergy Software).

In Planta Engineering. For trichome specific expression of *zFPS* and *ShZIS* we used the tomato Methyl Ketone Synthase (MKS1) promoter (5) for *zFPS* and the Monoterpene Synthase 1 (MTS1, AY840091) (6) for *ShZIS*. To establish that expression under these promoters was trichome specific, constructs with the uidA reporter (GUS) driven by the MKS1 or the MTS1 promoter were constructed. The Methyl-Ketone Synthase promoter (5) was cloned from genomic DNA of the wild tomato *S. habrochaites*.
PI126449. The 1733 bp fragment was used to drive GUS or zFPS in the pKG1662 vector of which the 35S-promoter was removed using HindIII and XbaI. A 1256 bp promoter fragment of MTS1 was obtained by performing a nested PCR using MTS1 specific primers on adapter ligated genomic DNA for S. lycopersicum Moneymaker. The MTS1 promoter was cloned into the pKG1662 vector driving GUS or ShZIS in the same way as described for MKS1. Promoter-GUS and promoter-zFPS/ShZIS-terminator cassettes were cloned into pBIN-plus (7). Constructs were transformed to Agrobacterium tumefaciens GV3101 and used to transform tomato S. lycopersicum Moneymaker (8). Plants were regenerated under kanamycin selection. The presence of terpenes was analyzed by SPME on ground leaf material or by injection of hexane leaf washes into the GC-MS. The 7-epizingiberene sterioisomer was confirmed on the B-DM column.

Analysis of promoter activity was carried out using histological GUS assays. Plant material was washed in staining buffer [phosphate buffer (40 mM K$_2$HPO$_4$/10 mM KH$_2$PO$_4$)/0.2% Triton X-100, pH 7.2] and incubated overnight at 37 °C in the presence of 1 mM X-gluc (β-D-glucuronide cyclohexylamine salt). Samples were destained with 70% (vol/vol) ethanol and tissue-specific expression of the promoters analyzed under a stereomicroscope.

Bioassays. A Bemisia tabaci (9) population was maintained in a climatised chamber (Snijders Tilburg; T 28 °C, 16-h light, RH 75%) on cucumber. F2 cuttings, S. habrochaites PI127826 and S. lycopersicum cv. Moneymaker and transgenic lines were grown in soil and kept in a climatised greenhouse compartment. Twenty adult whiteflies were anesthetized with CO$_2$, placed in a clipcage (2.5 cm diameter; Bioquip) and attached to a young leaf of Moneymaker. The MTS1 promoter was cloned into the pKG1662 vector of which the 35S-promoter was removed in the same way as described for MKS1. Promoter-GUS and promoter-zFPS/ShZIS-terminator cassettes were cloned into pBIN-plus (7). Constructs were transformed to Agrobacterium tumefaciens GV3101 and used to transform tomato S. lycopersicum Moneymaker (8). Plants were regenerated under kanamycin selection. The presence of terpenes was analyzed by SPME on ground leaf material or by injection of hexane leaf washes into the GC-MS. The 7-epizingiberene sterioisomer was confirmed on the B-DM column.

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Statistics. Statistical analyses were performed using ANOVA followed by the LSD test for comparison of individual means using the statistical program SPSS (IMB SPSS Inc. 2010). Levine’s test of equality of error variances was included and where necessary values were log-transformed before analyses.

Fig. S1. (A) Relative zingiberene content in leaves (%) of an F2 population of *S. lycopersicum* cv Moneymaker × *S. habrochaites* PI127826. Colored bars are zingiberene levels in selected F2 plants 141, 7, 71 and 2. Zingiberene is absent in *S. lycopersicum* and 100% in *S. habrochaites* PI127826. Zingiberene was measured in ground leaves by SPME. (B) Terpenes produced by F2 plants of an interspecific cross between *S. lycopersicum* cultivar Moneymaker and *S. habrochaites* PI127826. Germacrene B (black bars), zingiberene (red bars), total sesquiterpenes (dark gray bars), total monoterpenes (light gray bars) (ng/mg leaf FW), measured in a leaf wash of F2 plants and both parents (n = 5–8). Bars represent means ± SE. Note: monoterpenes levels in F2-2 are 4 times higher than presented.
Fig. S2. Toxicity of zingiberene to B. tabaci in F2 plants of an interspecific cross between S. lycopersicum cultivar Moneymaker and S. habrochaites PI127826 using clip cage assays. Correlation between 7-epizingiberene (ng/mg FW) in leaf washes in F2 plants with the total number of eggs on a leaf (A) and mortality (%) of adult whiteflies (B).
Fig. S3. (A) Alignment of tomato TPS class-e/f enzymes from *S. lycopersicum*, ShZIS from *S. habrochaites* PI127826, ShSBS from *S. habrochaites* LA1777. Alignments were made with CLUSTALW using the BLOSUM matrix (www.ch.embnet.org/software/ClustalW.html). Homology was visualized using Boxshade (www.ch.embnet.org/software/BOX_form.html). (B) Phylogenetic tree of (potentially) functional terpene synthase cDNAs of *S. lycopersicum* (Sl) and *S. habrochaites* (Sh) and zingiberene synthases (ZIS) of sorghum (Sb), rice (Os), grape (Vv), and basil (Ob). ND: activity not determined, Unidentified: product not identified. Alignments were made with CLUSTALW. The phylogenetic tree was constructed after bootstrap analysis (n = 1,000) using Lasergene DNAstar Megalign software (DNASTAR).
Fig. S4. 7-Epizingiberene production is trichome specific. (A) Tissue specific relative expression of zFPS (gray bars) and ShZIS (black bars) in S. habrochaites PI127826 determined by Q-RT-PCR. Bars represent means ± SE (n = 3). (B) Concentration of R-curcumene and 7-epizingiberene (ng/mg FW leaf) measured in 5 min hexane leaf wash in untreated plants (gray bars) and plants 24 h after jasmonic acid treatment (black bars). Bars represent means ± SE (n = 5). (C, D) Trichome-specific GUS expression in S. lycopersicum cv Moneymaker transformed with constructs containing GUS driven by the Methyl Ketone Synthase (MKS1) promoter (A) or by the Monoterpene synthase (MTS1) promoter (B). GUS activity was determined by staining with X-Gluc (16h).
Fig. S5. Trichomes on adaxial (A and C) and abaxial (B and D) leaf surface of: *S. lycopersicum* cv Moneymaker transformed with pMKS1-zFPS and pMTS1-ShZIS (A and B) *S. habrochaites* PI1217826 (C and D). (E) number of glandular trichomes type VI and IV on abaxial (gray bars) and adaxial (black bars) side of the leaf of Moneymaker, transgenic line 2 and *S. habrochaites* PI127826. Means ± SE (n = 5).
**Fig. S6.** *Tetranychus evansi* fecundity on *S. lycopersicum* cv Moneymaker, *S. lycopersicum* transgenic line 2 and *S. habrochaites* PI127826 displayed as number of eggs per mite per 4 d. Means ± SE (n = 60), different letters signify statistical differences (P < 0.05).

**Fig. S7.** Spider mite selection on 7-epizingiberene producing transgenic does not significantly improve performance. A total of 480 adult mites (*Tetranychus urticae*) were cultivated on *S. lycopersicum* cv Moneymaker (MM) or on *S. lycopersicum* transgenic line 2 for 5 wk (i.e., two full generation cycles) after which 200 adult mites of each were selected and allowed to produce eggs on fresh leaves (of the same genotype they had been selected on). After 48 h the adults were removed and their eggs allowed to develop into adults over a period of 14 d. These adult mites were used for the oviposition test depicted. Four combinations correspond to the four respective bars presented in the figure: (1) mites which had been selected on MM were allowed to produce eggs on MM leaf disks (MM -> MM with n = 60); (2) mites which had been selected on MM were allowed to produce eggs on Line 2 leaf disks (MM -> Line 2 with n = 60); (3) mites which had been selected on Line 2 were allowed to produce eggs on MM leaf disks (Line 2 -> MM with n = 60) and finally (4) mites which had been selected on Line 2 were allowed to produce eggs on Line 2 leaf disks (Line 2 -> Line 2 with n = 60). Bars represent mean number of eggs per mite per 4 d ± SE, different letters signify statistical differences (P < 0.05).
Fig. S8. (A) Effect of 7-epizingiberene in F2 tomato lines of an interspecific cross between *S. lycopersicum* cv Moneymaker and *S. habrochaites* PI127826 on the performance of the larvae of caterpillars (*Manduca sexta*, tobacco hornworm). Gray symbols: control plant (F2-200/2). Black symbols: F2 containing 7-epizingiberene (F2-100/21). Weight in mg, mean ± SE, *n* = 16 at the start of the assay, *P* < 0.001. (Right) Photos are *M. sexta* caterpillars and corresponding control and 7-epizingiberene producing F2 plants on which they fed (*t* = 10 d). (B) Effect of 7-epizingiberene in F2 tomato lines of an interspecific cross between *S. lycopersicum* cv Moneymaker and *S. habrochaites* PI127826 on the performance of neonates of *Leptinotarsa decemlineata* (Colorado Potato Beetle) in a choice assay between control plant (F2-200/2, gray bar) and a plant containing 7-epizingiberene (F2-100/21, black bar). Bars represent damage (%) to a leaf disk 48 h after start of the experiment, mean ± SE, *n* = 15, *P* < 0.01.

Table S1. Single nucleotide polymorphisms (SNPs) in ShZIS in five different *S. habrochaites* varieties all producing 7-epizingiberene

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Amino acid change | Arg (G) - Gln (A) | Ser (G) - Thr (C) | no | Met (G) - Ile (A) | no | Ala (C) - Val (T) | Met (G) - Ile (A) | no | Ile (A) - Leu (C) | Pro (G) - Leu (T) |
|-------------------|-------------------|-------------------|----|-------------------|----|-------------------|-------------------|----|-------------------|-------------------|

no, no amino acid change.