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Overexpression of the lemon basil α-zingiberene synthase gene increases both mono- and sesquiterpene contents in tomato fruit

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Summary

α-Zingiberene synthase (*ZIS*), a sesquiterpene synthase gene that was isolated from lemon basil (*Ocimum basilicum* L.), encodes an enzyme that catalyzes the formation of α-zingiberene, and other sesquiterpenes, from farnesyl diphosphate. Transgenic tomato fruits overexpressing *ZIS* under the control of the fruit ripening-specific tomato polygalacturonase promoter (*PG*) accumulated high levels of α-zingiberene (224–1000 ng g^{-1} fresh weight) and other sesquiterpenes, such as α-bergamotene, 7-*epi*-sesquithujene, β-bisabolene and β-curcumene, whereas no sesquiterpenes were detected in non-transformed control fruits. The *ZIS*-transgenic fruits also produced monoterpenes, such as α-thujene, α-pinene, β-phellandrene and γ-terpinene (1–22 ng g^{-1} fresh weight), which were either not detected or were found only in minute concentrations in control fruits. Recombinant ZIS overexpressed in *Escherichia coli* catalyzed the formation of these monoterpenes from geranyl diphosphate. As the ZIS protein apparently lacks a transit peptide, and is localized in the cytosol, the production of monoterpenes in the transgenic tomatoes suggests that a pool of geranyl diphosphate is available in the cytosol. The phenotype of the *ZIS*-transgenic tomatoes was the same as that for wild-type tomatoes, with regard to plant vigor and shape, but transgenic plants exhibited a small decrease in lycopene content. This study thus showed that the synthesis of both mono- and sesquiterpenes can be enhanced by the ectopic expression of a single transgene in tomato fruit, and it further demonstrated the interconnection between the pools of terpenoid precursors in the plastids and the cytosol.

Keywords: **α-zingiberene, monoterpenes, sesquiterpenes, tomato, volatiles, metabolic engineering.**

Introduction

Isoprenoids, compounds belonging to one of the most diverse families of plant metabolites, play important roles in plant membrane structure, redox chemistry, growth regulation, signaling and defense mechanisms (Dudareva *et al.*, 2004; Pichersky and Gershenzon, 2002; Pichersky *et al.*, 2006). Despite their diversity, isoprenoids are derived from common building units: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP; Croteau and Karp, 1991; Croteau *et al.*, 2000; McGarvey and Croteau, 1995). In plants, IPP is synthesized via two parallel pathways: the mevalonate (MVA) pathway in the cytosol and the methylerythritol 4-phosphate (MEP) pathway in the plastids (Lichtenthaler, 1999; Rodriguez-Concepcion and Boronat, 2002; Rohdich *et al.*, 2003; Rohmer, 2003). Both the MVA and the MEP pathway give rise to DMAPP, in addition to IPP, and both the cytosolic and the plastidic compartments contain an enzyme – isopentenyl diphosphate isomerase (IDI) – that reversibly converts IPP to DMAPP (Phillips *et al.*, 2008). In

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the plastids, one IPP molecule and one DMAPP molecule are combined by geranyl diphosphate synthase (GPPS) to produce geranyl diphosphate (GPP), which serves as the precursor for various monoterpenes. In addition, geranylgeranyl diphosphate (GGPP) is produced from three IPP molecules and one DMAPP molecule by geranylgeranyl diphosphate synthase (GGPPS); GGPP serves as the precursor for diterpenes such as gibberellins, side-chains of chlorophyll and tocopherols and tetraterpenes, such as the carotenoids. In the cytosol, two molecules of IPP are combined with one molecule of DMAPP by farnesyl diphosphate synthase (FPPS) to form farnesyl diphosphate (FPP), which is the precursor for the synthesis of sesquiterpenes and phytosterols (Lichtenthaler, 1999; Rodriguez-Concepcion and Boronat, 2002; Rohdich *et al.*, 2003; Rohmer, 2003). A group of enzymes known as terpene synthases convert the respective prenyl diphosphate precursors to the different mono-, sesqui- and diterpene skeletons that undergo subsequent modifications. Terpene synthases often have the ability to generate multiple products from the same prenyl diphosphate precursor (e.g. Portnoy *et al.*, 2008; Steele *et al.*, 1998; Tholl *et al.*, 2005).

The tomato (*Solanum lycopersicum* L.) fruit is rich in terpenoids. During early fruit development, the cytosolic pathway is operational, producing the glycoalkaloids and sterols that are essential for fruit development and ripening (Gaffe *et al.*, 2000; Gillaspy *et al.*, 1993; Jelesko *et al.*, 1999; Narita and Gruissem, 1989). Young, green fruits have trichomes that also synthesize a variety of volatile terpenes (Li *et al.*, 2004), but these trichomes dry up and fall off as the fruit matures. During ripening, the activity of the plastidic terpenoid pathway increases, leading mainly to the accumulation of the red tetraterpene pigment lycopene and other carotenoids (Ronen *et al.*, 1999). However, ripe tomato fruits contain only minute quantities of monoterpenes and sesquiterpenes (Buttery *et al.*, 1971, 1987, 1990; Petro-Turza, 1987), which are important contributors to the aroma and fragrance of many fruit and floral species (Croteau *et al.*, 2000; Dudareva *et al.*, 2004; Pichersky *et al.*, 2006; Schwab *et al.*, 2008).

The ability to genetically engineer the tomato plastidic terpenoid pathway to produce monoterpenes has been shown by the ectopic expression of the *Clarkia breweri* linalool synthase (*LIS*) gene under the control of the tomato *E8* promoter (Lewinsohn *et al.*, 2001), and by the ectopic expression of the lemon basil geraniol synthase (*GES*) gene under the control of the fruit ripening-specific tomato polygalacturonase (*PG*) promoter (Davidovich-Rikanati *et al.*, 2007). The expression of *LIS* in tomatoes led to the production and accumulation of the aroma compounds *S*-linalool and 8-hydroxylinalool, in ripening fruits, without adversely affecting the accumulation of carotenoids. The expression of *GES* resulted in the accumulation of high levels of geraniol, and of more than ten geraniol derivatives. The accumulated geraniol and derivatives had a profound impact on tomato flavor and aroma, as shown by organoleptic evaluations (Davidovich-Rikanati *et al.*, 2007). However, in *GES* transgenic fruits, the levels of lycopene decreased by 50% as compared with controls.

In contrast to the two above-described successful attempts to engineer monoterpene biosynthesis in the plastids of tomato fruits, engineering the synthesis of sesquiterpenes has not been reported in tomato fruit. As an FPP synthase gene is expressed during the late stages of tomato fruit development (Gaffe *et al.*, 2000), it is indeed possible that the concentration of FPP in the ripe fruit could support the synthesis of volatile sesquiterpenes. We therefore attempted to produce sesquiterpenes by transforming tomato with the α-zingiberene synthase (*ZIS*) gene, previously isolated from basil (*Ocimum basilicum* L.). This gene has been shown to encode a terpene synthase enzyme that catalyzes the *in vitro* formation of a number of sesquiterpenes, including α-zingiberene as the major product from FPP (Iijima *et al.*, 2004). Overexpression of the *ZIS* gene under the control of the tomato *PG* promoter was directed to the post-breaker (Br) stage, during which almost no sesquiterpenes are produced in wild-type tomatoes. Here, we report that the ectopic expression of *ZIS* in tomato fruit led to the formation of 15 sesquiterpenes, and to the unexpected accumulation of 10 additional monoterpenes.

Results

Volatile sesquiterpene profiles of transgenic tomatoes expressing ZIS

To test the possibility of diverting the tomato cytosolic terpenoid pathway, and the availability of FPP for the production of volatile sesquiterpenes, tomato plants were transformed with a construct harboring lemon basil *ZIS* cDNA coupled to the PG promoter. The presence of *ZIS* in the first generation of kanamycin-resistant regenerated tomato plants (T_1) was determined by PCR and Southern blot analysis. Several transgenic lines that contained between one and three copies of *ZIS* were identified (data not shown).

Analysis of the volatiles present in ripe fruits indicated that all three parental transgenic lines accumulated α-zingiberene (100–700 ng g^{-1} fresh weight, FW), whereas α-zingiberene was absent in all of the non-transgenic fruits (data not shown). The transgenic line with the highest concentration of α-zingiberene (700 ng g^{-1} FW) was self-crossed to produce T_2 plantlets. The latter were screened by PCR for the presence of *ZIS*, and 10 positive progenies were grown to fruit set.

Analysis of volatiles in the ripe fruit showed marked differences in sesquiterpene profiles between the T_2

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Table 1 Concentrations of volatiles in wild type (WT) and *ZIS*-transgenic tomato fruit

		Concentration of volatiles (ng g^{-1} fresh weight)*				
Group	Compound	WT	Line 2–2	Line 2–6	Line 2–10	Line 2–21
Sesquiterpenes	*7-epi*-sesquithujene	0	153 (±6)	112 (±24)	31 (±0.3)	92 (±3)
	sesquithujene	0	87 (±4)	69 (±13)	21 (±1)	61 (±4)
	cis-α-bergamotene	0	91 (±2)	87 (±22)	24 (±0.1)	63 (±7)
	trans-α-bergamotene	0	244 (±2)	210 (±62)	41 (±2)	144 (±8)
	cis-β-farnesene	0	40 (±16)	0	11 (±1)	14 (±4)
	trans-β-farnesene	0	230 (±5)	107 (±37)	29 (±2)	82 (±5)
	α-acoradiene	0	116 (±15)	78 (±26)	31 (±9)	78 (±23)
	curcumene AR	0	49 (±2)	99 (±33)	17 (±0.4)	85 (±13)
	α-zingiberene	0	1012 (±2)	719 (±150)	224 (±10)	635 (±12)
	β-bisabolene	0	148 (±20)	117 (±40)	32 (±3)	89 (±7)
	β-curcumene	0	91 (±13)	92 (±31)	22 (±0.2)	67 (±17)
	β-sesquiphellandrene	0	138 (±13)	96 (±27)	37 (±1)	117 (±21)
	trans-γ-bisabolene	0	26 (±0.5)	32 (±6)	13 (±1)	39 (±2)
	cis-γ-bisabolene	0	15 (±1)	19 (±5)	4 (±0.2)	16 (±1)
	Unidentified sesquiterpene	0	43 (±10)	33 (±6)	23 (±3)	54 (±6)
Monoterpenes	α-thujene**	0	8 (±2)	9 (±0.5)	6 (±0.1)	12 (±0.2)
	α-pinene**	1 (±0.5)	22 (±5)	16 (±0)	16 (±0.1)	22 (±0.2)
	sabinene**	0	0	2 (±0.2)	1 (±0.2)	1 (±0)
	β-pinene	0	5 (±1)	6 (±0.1)	2 (±0)	2 (±0)
	β-myrcene**	0	3 (±0.2)	3 (±0)	2 (±0.2)	3 (±0.3)
	δ-2-carene**	8 (±2)	11 (±2.5)	5 (±4)	8 (±0.2)	9 (±0)
	α-phellandrene**	0	1 (±0.1)	1 (±0.7)	1 (±0.1)	1 (±0.2)
	α-terpinene**	0	4 (±0.5)	2 (±0.2)	3 (±0.4)	5 (±2)
	p-cymene	2 (±2)	8 (±0.5)	8 (±2.4)	4 (±0.1)	11 (±0)
	limonene	12 (±4)	19 (±2)	18 (±3)	8 (±0.3)	15 (±0)
	β-phellandrene	11 (±5)	21 (±4)	29 (±8)	14 (±4)	24 (±0)
	trans-β-ocimene**	0	1 (±0)	1 (±0.8)	1 (±0.1)	1 (±0)
	γ-terpinene**	0	10 (±3)	7 (±2)	7 (±0.5)	14 (±2)
	terpinolene**	0	2 (±0.1)	0	2 (±0.1)	3 (±1)
	terpinen-4-ol	0	21 (±2.5)	25 (±3.6)	5 (±1)	15 (±3)
	neral	12 (±5)	10 (±1)	11 (±2)	7 (±0)	18 (±4)
	geranial	106 (±34)	54 (±2)	54 (±12)	46 (±2)	146 (±14)
Norisoprenes	6-methyl-2-hepten-1-one	109 (±68)	70 (±14)	91 (±21)	52 (±1)	221 (±10)
	β-cyclocitral	0	6 (±4)	11 (±2)	0	0
	2,3-epoxygeranial	45 (±14)	25 (±3)	23 (±5)	22 (±1)	59 (±13)
	geranyl acetone	38 (±12)	19 (±5)	79 (±15)	17 (±3)	38 (±0.1)
	β-ionone	16 (±7)	11 (±2)	31 (±5)	8 (±0.5)	22 (±0)
	dihydroactinodiolide	21 (±8)	24 (±5)	31 (±6)	15 (±0.2)	28 (±5)
	trans-psi-ionone	27 (±11)	17 (±1)	16 (±4)	13 (±1)	40 (±0.2)
Others	isobutylthiazole	105 (±26)	107 (±24)	107 (±13)	87 (±3)	214 (±0)
	guaiacol	838 (±145)	283 (±54)	1068 (±93)	185 (±51)	648 (±30)
	phenyl ethyl alcohol	73 (±37)	43 (±12)	123 (±3)	54 (±11)	28 (±2)
	methyl salycilate	991 (±168)	508 (±130)	1489 (±171)	471 (±98)	867 (±16)
	2-methoxy-4-vinylphenol	85 (±28)	17 (±2)	71 (±14)	12 (±2)	128 (±14)
	eugenol	143 (±113)	85 (±22)	132 (±1)	82 (±13)	63 (±11)
	benzaldehyde	38 (±13)	4 (±1)	15 (±2)	8 (±4)	13 (±0.5)

*Values represent the means of two (±SE) independent determinations of ripe fruits (10 days post 'breaker' stage). Similar compositions were obtained for two independent parental lines and 10 more progeny lines (not shown).

**Monoterpene content was evaluated by solid-phase micro extraction (SPME) analysis, as most monoterpenes are not detected in methyl *tert*-butyl ether (MTBE) extracts.

transgenic plants and the non-transformed controls (Table 1). Ripe transgenic fruits accumulated high levels of α-zingiberene (200–1000 ng g^{-1} FW), in addition to various quantities (5–250 ng g^{-1} FW) of other sesquiterpenes that were not detected in ripe control fruits. The sesquiterpenes, 7-*epi*-sesquithujene, *trans*-α-bergamotene, β-bisabolene and β-sesquiphellandrene accumulated in levels averaging at 90–250 ng g^{-1} FW, whereas sesquithujene, *cis*-α-bergamotene, *cis*- and *trans*-β-farnesene, α-acoradiene, curcumene AR, β-curcumene, *cis*-γ-bisabolene and

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trans-γ-bisabolene accumulated in smaller quantities of 5–90 ng g^{-1} FW (Table 1). Only trace levels of 3-*cis*-6-*trans*-α-farnesene and 3-*cis*-6-*cis*-α-farnesene were found (not shown).

ZIS expression and sesquiterpene synthase activity in transgenic tomatoes during ripening

To facilitate a better understanding of the biochemical and molecular basis of the accumulation of sesquiterpenes in the transgenic fruits, we examined *ZIS* expression and total sesquiterpene synthase activities in crude extracts of fruits over the course of fruit development (Figure 1). As expected from an expression pattern directed by the *PG* promoter, there was no apparent expression of *ZIS* at the mature green (MG) stage (Figure 1b). The *ZIS* transcript in the transgenic tomatoes was first detected at the Br stage. Expression of *ZIS* reached the highest level 3 days after the Br stage (Br + 3), and declined in ripe red fruit (from Br + 8 to Br + 10; Figure 1b). We also evaluated the total sesquiterpene synthase activity in wild-type and *ZIS* transgenic tomato fruits by incubating a radioactively labeled FPP substrate with cell-free fruit extracts, and then measuring the total radioactivity in the hexane fraction. Crude extracts of both wild-type and transgenic fruits at the MG stage exhibited about 500 pkat g^{-1} FW, and activity dropped in both types of fruit at the Br stage. However, although the total sesquiterpene synthase activity continued to drop in the wild-type fruit as the fruit matured, the total sesquiterpene synthase activity in the *ZIS* transgenic fruits was dramatically increased as ripening proceeded, and reached its highest levels at Br + 3. At this point, the total sesquiterpene synthase activity in the transgenic ripe fruits was sixfold higher than that in the control fruit (Figure 1c).

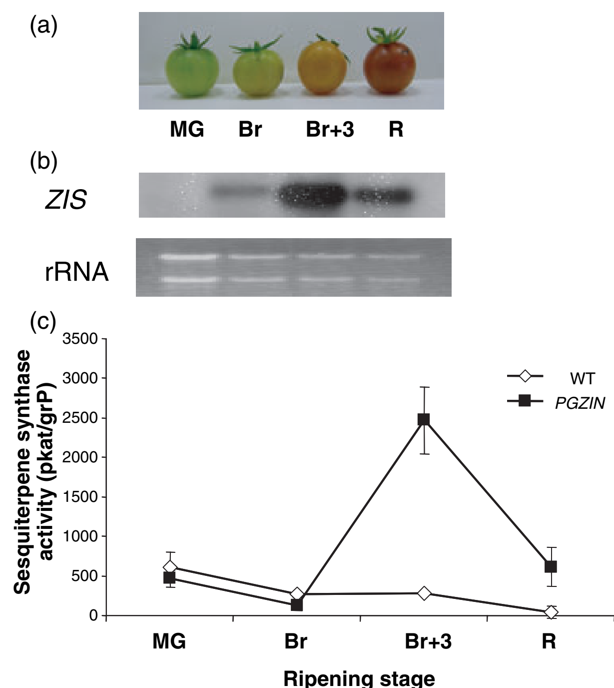

Figure 1. Time course of *ZIS* expression and sesquiterpene synthase activity in *ZIS* transgenic and control fruits over the course of ripening. Tomatoes were harvested at four different ripening stages, as indicated.
(a) Phenotypes of transgenic fruits during ripening.
(b) Northern blot analysis of *ZIS* transcript in transgenic fruits during ripening. Each lane contained 5 μg of transgenic fruit total RNA.
(c) Sesquiterpene synthase activity in control and transgenic fruits during ripening. Each data point represents an average of two repeats of the analysis of line 2–2. Similar results were obtained for analysis of other transgenic lines.

The main sesquiterpenes produced by the cell-free extracts derived from Br + 3 transgenic fruits were α-zingiberene, 7-*epi*-sesquithujene, sesquithujene, *cis*- and *trans*-α-bergamotene, β-bisabolene, β-sesquiphellandrene, *cis*- and *trans*-β-farnesene, α-acoradiene, curcumene AR, β-curcumene, *cis*-γ-bisabolene, *trans*-γ-bisabolene and farnesol, with trace levels of 3-*cis*-6-*trans*-α-farnesene and 3-*cis*-6-*cis*-α-farnesene (Figure 2a). The volatiles produced by the cell-free extracts derived from the Br + 3 control tomatoes incubated with FPP revealed that the endogenous activity produced β-farnesene, 3-*cis*-6-*trans*-α-farnesene, 3-*cis*-6-*cis*-α-farnesene and farnesol from FPP (Figure 2b). As previously reported, basil ZIS purified from *Escherichia coli* and incubated with FPP catalyzed the formation of α-zingiberene as well as the formation of 7-*epi*-sesquithujene, sesquithujene, *cis*- and *trans*-α-bergamotene, *cis*- and *trans*-β-farnesene, α-acoradiene, curcumene AR, 3-*cis*-6-*trans*-α-farnesene, 3-*cis*-6-*cis*-α-farnesene, β-bisabolene, β-curcumene, *cis*-γ-bisabolene, β-sesquiphellandrene and *trans*-γ-bisabolene (Figure 2c).

Accumulation of monoterpenes in ZIS transgenic tomatoes

In addition to the accumulation of ZIS-catalyzed sesquiterpenes in the *ZIS* transgenic fruits, a marked increase in monoterpene levels was also observed in the three parental transgenic lines (not shown), and in the T_2 progenies analyzed (Table 1). α-Pinene, α-thujene, *p*-cymene, γ-terpinene and terpinen-4-ol were detected at levels of 5–29 ng g^{-1} FW in the transgenic fruits, but not (or only in minute levels) in non-transgenic fruits. The levels of limonene, β-phellandrene, sabinene, β-pinene, β-myrcene, α-phellandrene, α-terpinene and β-ocimene also increased in transgenic tomatoes, as compared with controls.

To examine the biochemical basis of monoterpene accumulation in transgenic fruits, we evaluated total monoterpene synthase activity at various stages of fruit development (Figure 3a). The levels of the total monoterpene synthase activity were similar in control and *ZIS* transgenic fruits at the MG stage, probably because of endogenous monoterpene synthase activity (Figure 3a). In the *ZIS* transgenic tomatoes, monoterpene synthase activity increased as

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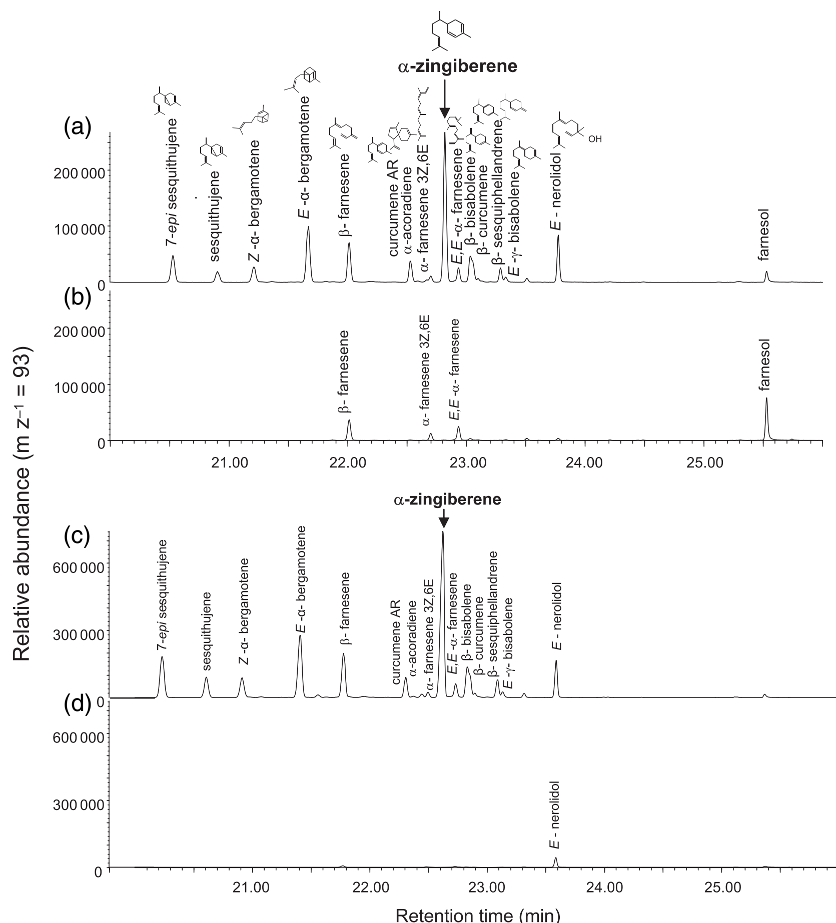

Figure 2. Identification of volatiles produced by cell-free extracts derived from transgenic and control tomatoes, and by the recombinant ZIS activity overexpressed in *Escherichia coli*.
Gas chromatographic separation of products synthesized *in vivo* by sesquiterpene synthase activity in crude protein extracts isolated from breaker + 3 (Br + 3) fruits of *ZIS* transgenic (a) and control fruit (b). Gas chromatographic separation of products synthesized *in vitro* by (c) *ZIS*-overexpressing *E. coli* lysates and (d) control *E. coli* protein extracts incubated with farnesyl diphosphate (FPP) as substrate. Reaction products were obtained by solid-phase micro extraction (SPME). The trace obtained for $m/z = 93$ typical for these sesquiterpenes is shown. Each compound was identified by comparisons of retention times and mass spectra of authentic standards taken from MS libraries. Identified sesquiterpenes are labeled. Reactions with boiled protein extracts incubated with FPP as substrate, and reactions containing no Mg^{2+}/Mn^{2+} as co-factors, were used as controls that did not produce terpenes (data not shown).

ripening proceeded, and reached its highest level at Br + 3, whereas in control fruits the monoterpene synthase activity decreased, and became very low after the Br stage (Figure 3a). Crude protein extracts of transgenic fruits at the Br + 3 stage incubated with GPP produced a larger array of volatiles than that synthesized in control fruits; these volatiles included α-thujene, α-pinene, sabinene, β-pinene, β-myrcene, α-phellandrene, α-terpinene, limonene, β-phellandrene, β-ocimene, γ-terpinene, terpinolene and nerol (Figure 3b). The products generated from the incubation of control extracts with GPP were β-myrcene, limonene, ocimene and geraniol (Figure 3c). However, we cannot rule out the possibility that geraniol was generated by the action of phosphatases on GPP, and that β-myrcene, limonene and ocimene are chemical derivatives of geraniol, as these terpenes were also detected when a geraniol standard was incubated with buffer only, without protein (data not shown). Our data suggested that ZIS could have monoterpene synthase activity in addition to its documented sesquiterpene synthase activity (Iijima *et al.*, 2004). To test this possibility, we determined the ability of recombinant ZIS to accept GPP as a substrate *in vitro.* It was indeed found that recombinant ZIS utilized GPP to produce the monoterpenes α-thujene, α-pinene, sabinene, β-pinene, β-myrcene, α-phellandrene, α-terpinene, limonene, β-phellandrene, β-ocimene, γ-terpinene, terpinolene and nerol (Figure 3d). None of these products are produced in lysates from *E. coli* cells devoid of the *ZIS* construct (Figure 3e).

Other changes in ZIS transgenic tomatoes

To evaluate whether the production of new sesquiterpenes and monoterpenes affected the accumulation of the main end products of terpenoid pathways, the levels of sterols and carotenoids were analyzed (Figure 4 and Table 2). No differences in stigmasterol, γ-sitosterol and β-amyrin were noted between ripe transgenic and wild-type tomatoes (Figure 4). Small decreases (between the experimental error values) in lycopene and phytoene contents were found in some of the transgenic lines tested, compared with controls (Table 2), and no clear correlation between carotenoid levels and volatile accumulation was noted. No phenotypic differences, including differences in plant vigor and shape, were found between the *ZIS* transgenic plants and wild-type plants.

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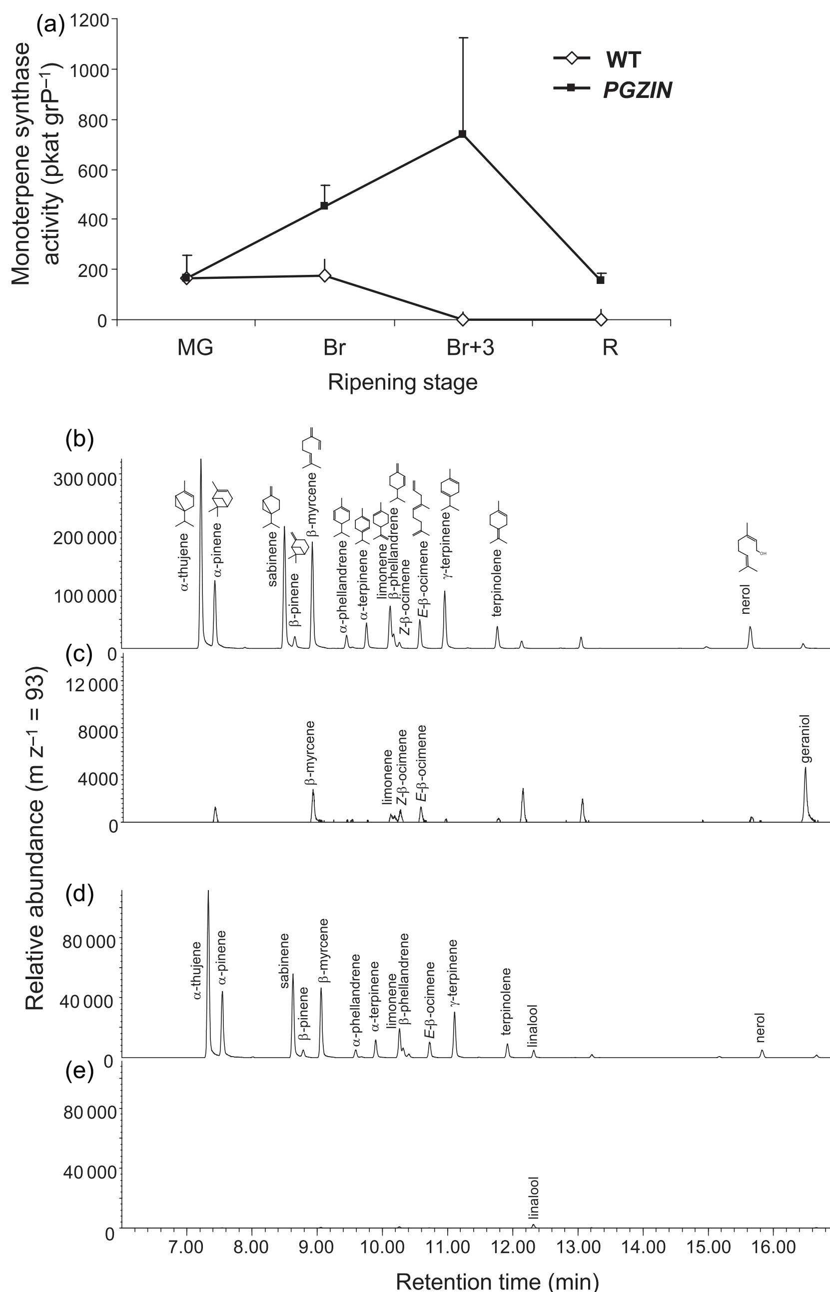

Figure 3. Time course of monoterpene synthase activity and its products in *ZIS* transgenic fruits and controls during ripening. Tomatoes were harvested at four different ripening stages, as indicated.
(a) Monoterpene synthase activity in control and transgenic fruits during ripening. Each data point represents an average of two repeats of the analysis of line 2–2.
(b) Gas chromatographic separation of products synthesized *in vitro* by monoterpene synthase activity in crude protein extracts isolated from breaker +3 (Br + 3) fruits of *ZIS* transgenic and control (c) fruit.
(d) Gas chromatographic separation of products synthesized *in vitro* by *ZIS*-overexpressing *Escherichia coli* lysates and control *E. coli* protein extracts (e) incubated with geranyl diphosphate (GPP) as substrate. Reaction products were obtained by solid-phase micro extraction (SPME) from GPP as a substrate. The trace obtained for $m/z = 93$, typical for monoterpenes, is shown. Each compound was identified by comparisons of retention times and mass spectra of authentic standards taken from MS libraries. The identified monoterpenes are labeled.

Discussion

Expression of ZIS in the cytosol of cells of ripening tomato fruit led to the synthesis of new sesquiterpenes

The ectopic expression of the lemon basil *ZIS* gene under the control of the tomato polygalacturonase promoter, led to the production of sesquiterpenes (up to ~2000 ng g^{-1} FW) in tomato fruit (Table 1). The sesquiterpenes included α-zingiberene, 7-*epi*-sesquithujene, α-bergamotene, β-bisabolene and β-sesquiphellandrene, which were not detected in controls. The successful synthesis of large quantities of sesquiterpenes in the *ZIS* transgenic fruit indicates that there was ample cytosolic supply of FPP, the product of the reaction catalyzed by FPPS, an enzyme known to be active in the ripening fruit of tomato (Gaffe *et al.*, 2000). No significant differences were noted in the contents of the main sterols stigmasterol, γ-sitosterol and β-amyrin, which are also products of the cytosolic terpenoid pathway, between transgenic fruits and control fruit (Figure 4). Curiously, however, although the levels of other FPP-derived compounds in the cytosol were not affected (Figure 4), a minor

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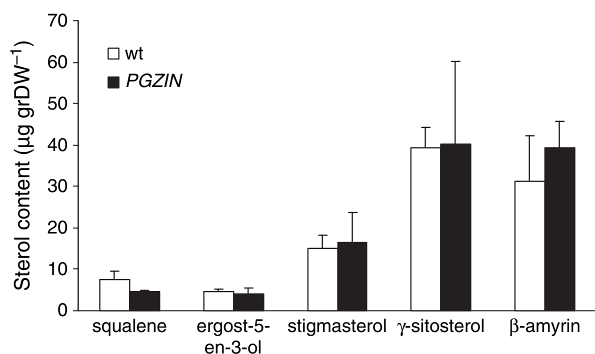

Figure 4. Concentrations of the main sterols and triterpenes in ripe transgenic fruits (*PGZIN*) and controls (wild type, wt). Values represent the means of two (±SE) independent determinations of ripe fruits (10 days post 'breaker' stage).

Table 2 Carotenoid content in wild-type and *ZIS*-transgenic tomato fruits

Line tested	Lycopene (μg g^{-1} FW)	β-Carotene (μg g^{-1} FW)	Phytoene (μg g^{-1} FW)
Wild type	99 ± 0.4	4 ± 0	5 ± 0
2–31	81 ± 0.1	4 ± 0.3	3 ± 0.2
2–3	90 ± 8.1	3 ± 0.2	3 ± 0.2
2–5	88 ± 6.5	4 ± 0	5 ± 0.1
2–6	69 ± 8.1	4 ± 0.1	2 ± 0.1
2–21	94 ± 8.9	3 ± 0.1	4 ± 0.2

Values represent the means (±SE) of two independent determinations of ripe fruits (10 days post 'breaker' stage).

decrease in the levels of carotenoids in the plastids was observed (Table 2), although to a much lower extent than that observed when the monoterpene synthase GES was expressed in the plastids of mature tomato fruit cells (Davidovich-Rikanati *et al.*, 2007). The small reduction in the contents of carotenoids in transgenic tomatoes expressing *ZIS* may be related to the production of the monoterpenes in these fruits, which indicates that some GPP from the plastidic terpenoid pathway was utilized for ZIS activity in the cytosol.

The overproduction of volatile sesquiterpenes has also been successfully engineered in Arabidopsis and tobacco. Overexpression of the maize *TPS10* gene, which codes for a sesquiterpene synthase, in *Arabidopsis thaliana* resulted in the plants producing sesquiterpene products identical to those released by maize, thus rendering the transgenic *A. thaliana* attractive to the natural enemies of maize pests (Schnee *et al.*, 2006). Production of the sesquiterpenes in this case did not result in any other noticeable changes in the plant morphology. In another study, exceptionally high levels of sesquiterpene production were achieved in tobacco plants, by diverting the carbon flow, through overexpression in either cytosolic or plastidic compartments of an avian FPSS and an appropriate terpene synthase (Wu *et al.*, 2006). The strategy enabled the synthesis of the sesquiterpenes patchoulol and amorpha-4,11-diene. These manipulations had a marked effect on plant phenotype, as reflected in leaf chlorosis, vein clearing and a decrease in size, all of which indicated possible toxic effects of the new sesquiterpenes, or depletion of substrate pools for other important functions of the plant, such as chlorophyll, carotenoid and gibberellin biosynthesis.

Expression of ZIS also led to the overproduction of monoterpenes

The expression of *ZIS* in ripe tomato fruits also led to the unexpected enhanced accumulation of monoterpenes (Table 1). Most of the novel monoterpenes, including α-thujene, γ-terpinene and terpinolene, but not terpinene-4-ol, were also produced by the recombinant ZIS (expressed in *E. coli*), when incubated with GPP (Figure 3c). Therefore, the activity of ZIS would explain the enhanced monoterpene accumulation, provided that sufficient GPP is available. The patterns of *ZIS* expression and monoterpene synthase activity also support the conclusion that ZIS activity is the main contributor to monoterpene synthesis in the transgenic lines (Figure 3a). Since the *ZIS* gene sequence lacks a plastidic transit peptide, and is therefore present in the cytosol, the production of the monoterpenes in the transgenic tomatoes suggests that a pool of GPP is available in this compartment. This finding is consistent with those of previous reports in other species, as described below. Transgenic tobacco plants carrying a construct of the *Perilla frutescens* limonene synthase (*LS*) directed to the cytosol accumulated limonene (although in levels that were much lower than in plants expressing a plastid-directed LS; Ohara *et al.*, 2003). Similarly, the formation of shikonin, a naphtoquinone monoterpene conjugate for which GPP is a precursor, was reported to occur in the cytosol of *Lithospermum erythrorhizon* cells (Sommer *et al.*, 1995). In another report, a terpene synthase isolated from strawberry (FaNES1), and shown to possess both monoterpene (linalool) and sesquiterpene (nerolidol) synthase activity, was overexpressed in Arabidopsis (Aharoni *et al.*, 2004). Transgenic plants accumulated both linalool and nerolidol. In these experiments, GFP localization showed that the recombinant FaNES1 was restricted to the cytosol. Therefore, the researchers suggested that the enzyme can act both as a monoterpene synthase and as a sesquiterpene synthase in the cytosol, thereby supporting the production of both monoterpenes and sesquiterpenes.

Although GPP appeared to be available in the cytosol in our study, the source of this GPP was not clear. Cytosolic GPP synthase activity has never been unambiguously reported in tomato, although Bouvier *et al.* (2000) suggested that an Arabidopsis GPP synthase gene encodes two isoforms: one targeted to the plastids and the other to the

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cytosol. However, we cannot exclude the possibility that ZIS utilizes GPP produced by FPPS. Whereas GPP is an intermediate in the synthesis of FPP, it has been postulated that GPP remains bound in the active site of FPPS during biosynthesis. However, it could be that some of the GPP intermediate is released without completion of FPP synthesis, resulting in the *in vivo* formation of monoterpenes. So far, no significant data are available on GPP formation and putative GPP synthase genes in tomato fruits. Nevertheless, profound changes in plastid structure, including membrane reorganization, take place during differentiation from chloroplasts to chromoplasts in ripening fruit (Bruno and Wetzel, 2004; Summer and Cline, 1999). Futhermore, it has been observed that the lycopene deposited in tomato fruit chromoplasts is in a crystalline form, often deforming the chromoplast membrane (Pyke and Howells, 2002). Although the specificity of protein targeting into the chromoplasts is usually similar to the targeting to chloroplasts (Bruno and Wetzel, 2004; Summer and Cline, 1999), these major structural changes in membrane organization could affect the intraplastidial compartmentization of substrates, leading to the availability of GPP in the cytosol.

Why do tomato fruits produce such small quantities of volatile monoterpenes and sesquiterpenes?

Wild and cultivated tomatoes produce high levels of sesquiterpenes in the leaves (Colby *et al.*, 1998). The sesquiterpene content of tomato leaves varies considerably among species, with β-caryophyllene and α-humulene being widespread in *S. lycopersicum*, *Solanum pimpinellifolium*, *Solanum peruvianum*, *Solanum cheesmanii*, *Solanum chilense* and *Solanum chumielewski* (Kant *et al.*, 2004; Lundgren *et al.*, 1985). In *Solanum hirsutum*, α-zingiberene, is a major leaf oil sesquiterpene, and this trait has been associated with resistance to the silverleaf whitefly *Bemisia argentifolii* (Freitas *et al.*, 2002). Many monoterpenes, including β-phellandrene, limonene and terpinolene, have been found in the leaves of the domesticated tomato (*S. lycopersicum*), and many are also present in the leaves of wild tomato species (Kant *et al.*, 2004; Lundgren *et al.*, 1985). β-Pinene, β-myrcene and 2-carene are constitutively produced, whereas linalool and β-ocimene production is induced after spider mite feeding (Kant *et al.*, 2004). To date, two monoterpene synthases and a sesquiterpene synthase have been isolated from the leaves and trichomes of the cultivated tomato (Colby *et al.*, 1998; van Schie *et al.*, 2007).

Ripe tomato fruit generally lack sesquiterpenes, and accumulate very low levels of monoterpenes (Table 1); most of them are degradation products of lycopene (Lewinsohn *et al.*, 2005a,b). Tomatoes also have low mono- and sesquiterpene synthase activities (Figures 1 and 3). Still, when either mono- or sesquiterpene synthases are overexpressed in transgenic tomatoes, high levels of mono- or sesquiterpenes are produced, indicating an apparent availability of both FPP and GPP pools (this study; Lewinsohn *et al.*, 2001; Davidovich-Rikanati *et al.*, 2007). Recent BLAST searches on expressed sequence tags (ESTs) from tomato fruit pericarp revealed only a single unidentified candidate for a monoterpene synthase, and no candidates for sesquiterpene synthases. Thus, it seems that mono- and sesquiterpene synthases are limiting factors, and prevent higher rates of mono- and sesquiterpene accumulation in tomato fruit. As has been shown for tomato leaves, and many terpene-producing plants, it is possible that tomato fruit terpene synthases are also exclusively expressed in trichomes on the surface of the fruit. Indeed, it has been shown that various types of trichomes on immature (green) tomato fruit are absent upon fruit maturation (Li *et al.*, 2004). This finding implies that if there are any mono- and sesquiterpene synthases expressed in fruits, they are expressed in very low levels, and mainly during the immature phase of fruit development.

Further study of the crosstalk processes and the regulation of the terpenoid biosynthetic pathways in tomato – together with the availability of other genes coding for the formation of terpenoid metabolites – will ultimately enable metabolic engineering to improve the flavor and aroma of the fruits, as well as the resistance to pests and pathogens. The data presented here and in previous works imply that the localization and compartments suggested for enzymes and substrates involved in terpene synthesis should be re-examined. Moreover, our data imply that there is crosstalk between the cytosolic and the plastidial pathways in a way that enables the synthesis of monoterpenes in the cytosol, although this process was previously thought to be confined to the plastid.

Experimental procedures

Identification of volatiles produced by recombinant ZIS activity

The full-length cDNA fragment encoding *ZIS*, originally isolated from a library of lemon basil (*O. basilicum*) glandular trichomes (GenBank accession no. AY693646), was cloned into the pCRT7/CT-TOPO TA vector (Invitrogen, http://www.invitrogen.com), and was then transformed into an *E. coli* expression system for the expression of the recombinant enzyme (Iijima *et al.*, 2004). Bacterial *E. coli* cells overexpressing *ZIS* were lysed, and 100 μl of the lysates was incubated for 4 h at 30°C with 10 μM GPP or 10 μM FPP, in a final volume of 400 μl of buffer, containing 50 mM bis-tris, pH 6.9, 1 mM DTT, 10% (v/v) glycerol, 0.1 mM $MnCl_2$ and 10 mM $MgCl_2$. Volatile products were extracted by solid-phase micro extraction (SPME), as previously described (Iijima *et al.*, 2004).

Terpene synthase activity assays

For terpene synthase activity assays in transgenic tomatoes, fruits were harvested at four different ripening stages: MG, Br, Br + 3 and

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ripe (between Br + 8 and Br + 10). Fruit tissue (2 g FW) was ground in liquid N_2, and soluble proteins were extracted by the addition of 6 ml of extraction buffer containing 50 mM bis-tris, pH 6.9, 1 mM DTT, 10% (v/v) glycerol, 1% (w/v) polyvinylpyrolidone-40 and 1% (w/v) polyvinylpolypyrolidone. Monoterpene synthase activity was assayed by incubating 10 μl of the crude enzyme sample in a final volume of 100 μl of buffer, containing 50 mM bis-tris, pH 6.9, 1 mM DTT, 10% (v/v) glycerol, 0.1 mM $MnCl_2$, 10 mM $MgCl_2$ and 10 μM [1–^{3}H]GPP or 10 μM [1–^{3}H]FPP (specific activity 20 Ci mol^{-1}; American Radiolabeled Chemicals, http://www.arcincusa.com). The reaction mixture was overlaid with 1 ml of hexane, and was incubated for 60 min at 30°C. After incubation, samples were mixed briefly and centrifuged to separate the radiolabeled volatiles from the aqueous phase. To separate monoterpene and sesquiterpene produced by the terpene synthases from alcohols produced by phosphatase activity, the upper hexane layer was mixed with ~100 μg of silica gel, and was then centrifuged briefly. The upper hexane layer (650 μl) was placed in a scintillation vial containing 3 ml of Ultimagold nonaqueous scintillation fluid (Packard Bioscience, http://las.perkinelmer.com), and radioactivity was counted with a Tri Carb 2800TR liquid scintillation counter (Perkin-Elmer, http://las.perkinelmer.com). Boiled enzyme extracts were used as controls.

Vector construction and plant transformation

The *O. basilicum ZIS* coding region was cloned into the binary vector pBin19 that already contained the tomato fruit specific *PG* promoter (4.8 kb) and *PG* terminator (1.8 kb), and the kanamycin-resistance marker gene *NPTII*, driven by the *CaMV 35S* promoter (Nicholass *et al.*, 1995). The binary vector was introduced into *Agrobacterium tumefaciens* strain *EHA105*, which was used for transformation. Tomato MP–1 plants were transformed according to Barg *et al.* (1997), with 100 mg l^{-1} kanamycin for selection.

The presence of *ZIS* in the first generation of kanamycin-resistant tomato plants was analyzed by PCR and Southern blot using the *ZIS* full-length clone as a probe. Three transgenic lines contained between one and three copies of *ZIS* (data not shown). A preliminary analysis of the volatile composition in ripe fruit showed that all transgenic lines produced α-zingiberene in ripening fruits. Line–2 plants, displaying the highest levels of α-zingiberene, were selfed to obtain the T_2 generation. T_2 plantlets were screened by PCR for the presence of *ZIS*, and 10 positive progenies from each line were grown till fruit set.

Molecular analysis of transgenic plants

Genomic DNA was extracted from young leaf tissue according to the method described by Murray and Thompson (1980). About 100 mg of young leaf tissue was ground in liquid N_2 with a polypropylene tip, and was then extracted with 700 μl of extraction buffer [0.15 M sorbitol, 0.1 M Tris-HCl, pH 7.5, 2 mM EDTA, 0.83 M NaCl, 0.83% (w/v) cetyl trimethyl ammonium bromide (CTAB), 0.83% (w/v) *N*-lauroylsarcosine, 0.05 M sodium bisulfite]. Genomic DNA was screened by PCR for the presence of *ZIS* in transgenic plants using a set of primers specific to *ZIS*. The PCR reaction was performed with a ready mix (ABgene, http://www.abgene.com) containing 2 mM $MgCl_2$, 100 ng genomic DNA and 50 μM of the following primers: sense primer 5′-ACTTGGATCCATGGAGTCAAGAAGGTC-3′ and antisense primer 5′-TGACTAGTTTATATGGTGATATTCATGGGTTG-3′ in a total volume of 25 μl. Amplification was performed in an Eppendorf Mastercycler gradient (Eppendorf AG. 5331, http://www.eppendorf.com) PCR device under the following conditions: 4 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, followed by 10 min at 72°C.

Northern blot analysis was performed to determine *ZIS* expression in transgenic fruits during ripening. Total RNA was isolated at four stages of ripening: MG, Br, Br + 3 and ripe fruits (between Br + 8 and Br + 10). RNA was isolated using Genelute total RNA isolation kit (Sigma-Aldrich, http://www.sigmaaldrich.com) according to the manufacturer's protocol, and 5 μg was separated on 1% (w/v) agarose-formaldehyde gel, and was then blotted to Hybond-N+ nylon membranes (Amersham Biosciences, http://www.amersham.com). Membranes were hybridized overnight at 65°C with ^{32}P-labeled full-length *ZIS* as a probe (Ready-To-Go™ DNA Labeling Beads; Amersham Biosciences), and were washed twice at 65°C with 2× SSC and 0.1% SDS for 15 min, followed by two washes of 10 min with 0.1× SSC, 0.1% SDS at 65°C. The membranes were exposed to BioMaxMS film (Kodak, http://www.kodak.com) for 2 days.

Extraction of volatile metabolites from tomato fruits

Approximately 30 g of fresh ripe fruits (between Br + 8 and Br + 10) were cut into small pieces and extracted with 100 ml of methyl *tert*-butyl ether (MTBE) by vigorous shaking on a shaker apparatus overnight. Each sample was supplemented with 100 μg of isobutyl benzene as an internal standard. The ethereal phase was separated off, dried with anhydrous Na_2SO_4 and concentrated to 2 ml under gentle N_2 flow. For SPME, 5 g of fresh ripe fruits (between Br + 8 and Br + 10) were cut into small pieces and placed in a 20-ml DuPont autosampler vial (DuPont Performance Elastomers, http://www.dupontelastomers.com) with a white solid-top polypropylene cap (Alltech, http://www.alltech.com). Samples were overlaid with 5 ml NaCl (25%) solution and 1 g NaCl (for inhibition of enzyme activity). Each sample was supplemented with 0.4 μg of 2-heptenone and 0.4 μg of longifolene as internal standards. Samples were incubated at room temperature (25°C) for 1 h, and thereafter volatile compounds were collected with an SPME device PDMS-100 with a polydimethylsiloxane fiber (Supelco, http://www.sigmaaldrich.com/Brands/Supelco_Home.html) by inserting the fiber into the tube and leaving it in place for 20 min at room temperature. After this incubation step, the SPME fiber was injected directly into the GC-MS.

GC-MS analysis of plant volatiles

A 1-μl aliquot of the concentrated MTBE extract was injected into a GC-MSD system (Agilent, http://www.home.agilent.com). The instrument was equipped with an Rtx–5 SIL column (30-m long × 0.25-mm inner diameter, 0.25-μm film thickness and stationary phase 95% dimethyl–5% diphenyl polysiloxane). Helium (0.8 ml min^{-1}) was used as a carrier gas with splitless injection. The injector temperature was 250°C, and the detector temperature was 280°C. The following conditions were used: initial temperature, 50°C for 1 min, followed by a ramp from 50 to 260°C at a rate of 5°C min^{-1}. A quadrupole mass detector with electron ionization at 70 eV was used to acquire the MS data in the range of 41 to 350 *m*/*z*. A mixture of straight-chain alkanes (C7–C23) was injected into the column under the aforementioned conditions for determination of retention times. The identification of the volatiles was assigned by comparison of their retention times with those of literature, and by comparison of spectral data with standards. The quantity of component in each sample was calculated as (peak area × internal standard response factor) divided by (response factor × internal standard peak area).

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Determination of carotenoid levels

Frozen samples of ripe fruit (between Br + 8 and Br + 10) were homogenized with a polytron apparatus, and 0.5 g of the homogenate was extracted with hexane:acetone:ethanol (50:25:25 v/v), which was followed by 5 min of saponification in 8% (w/v) KOH. The saponified material was extracted twice with 4 ml of hexane, which was then evaporated off under vacuum. The solid pellet was resuspended in 400 μl of acetonitrile:methanol:dichloromethane (45:5:50 v/v) and was then passed through a 0.2-μm nylon filter for HPLC analysis. Samples were analyzed in an Alliance photodiode array HPLC machine (Hewlett-Packard, http://welcome.hp.com) utilizing the gradient system described previously (Tadmor *et al*., 2000). The levels of lycopene, β-carotene and phytoene were quantified by comparison with calibration curves obtained with authentic standards.

Determination of sterol levels

Approximately 100 g of fresh ripe fruits (between Br + 8 and Br + 10) were ground in liquid N_2 and were then freeze-dried in a lyophilizer. Dry samples of 10 mg were extracted with petroleum ether in 40–60°C using a Soxhlet apparatus. The resulting oil was resuspended in 1 ml of hexane containing 10 μg of isobutyl benzene as an internal standard. A 1-μl aliquot of the concentrated extract was injected into a GC-MSD system (Agilent).

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