

Enrichment of tomato flavor by diversion of the early plastidial terpenoid pathway

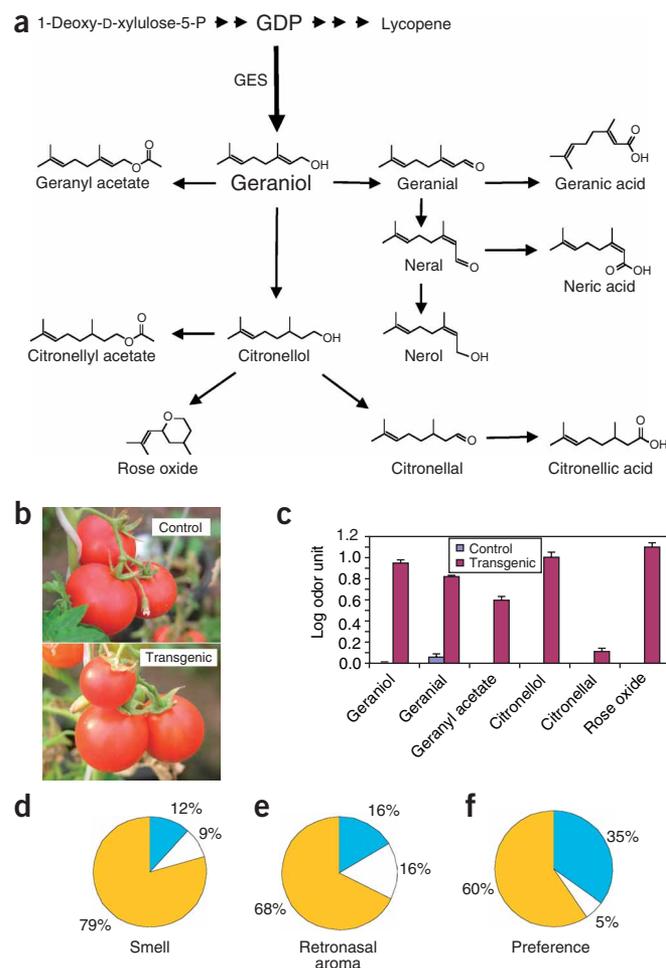
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We have modified the flavor and aroma of tomatoes by expressing the *Ocimum basilicum* geraniol synthase gene under the control of the tomato ripening-specific polygalacturonase promoter. A majority of untrained taste panelists preferred the transgenic fruits over controls. Monoterpene accumulation was at the expense of reduced lycopene accumulation. Similar approaches may be applicable for carotenoid-accumulating fruits and flowers of other species.

Selection for important agricultural traits often results in the loss of aroma and taste in tomato and other crops¹. Although > 400 different volatiles have been found in the tomato, only a few of them have a marked impact on its organoleptic properties^{2,3}. Thus, although metabolic engineering theoretically offers an ideal solution to improving the taste and aroma in tomato, this technique has thus far had only limited impact^{4–6}.

Figure 1 Diversion of the plastidial terpenoid pathway in tomato fruit. (a) Metabolism of geraniol in *GES*-expressing tomatoes. The geraniol formed was further metabolized by endogenous enzymes. (b) Phenotypes of control and transgenic fruits. (c) Contribution of geraniol derivatives to the aroma of transgenic tomatoes (in log odor values). Whereas log odor units are usually calculated based on headspace volatile analysis, they are used here to illustrate the relative difference between controls and transgenic lines. Means of four independent lines \pm s.e.m. are shown. (d–f) Organoleptic evaluations of *GES*-transgenic tomatoes. Smell (orthonasal route) evaluations by untrained panelists ($n = 34$) (d), retronasal aroma evaluation ($n = 37$) (e), preference tests ($n = 37$) (f). Orange color represents higher scores in transgenic fruit, blue represents higher scores in controls and white represents no differences in scores. Differences are significant by one-tailed paired *t*-tests ($P < 0.05$). GDP, geranyl diphosphate; *GES*, geraniol synthase.

Monoterpenes, which are important contributors to many fruit and floral scents⁷, are synthesized from geranyl diphosphate (GDP), an intermediate in carotenoid biosynthesis. Although the carotenoid pathway is highly active in ripening tomato fruits, leading to the production of lycopene⁸, ripe fruits contain only minute amounts of monoterpenes^{2,3}. To enhance monoterpene content, we expressed the lemon basil (*Ocimum basilicum* L. cv. Sweet Dani) geraniol synthase (*GES*) gene⁹ under the control of the tomato polygalacturonase promoter¹⁰. *GES* catalyzes the conversion of GDP to geraniol⁹, an acyclic monoterpene alcohol with an intense rose scent¹¹. Geraniol is



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Table 1 Concentrations of volatiles and carotenoids in *GES*-transgenic lines

Compound group	Compound	Line tested				
		MP-1 control	12-20	12-22	17-8	17-18
		Concentration (ng/g FW)				
Geraniol and derivatives	Geraniol	0	353	321	332	264
	Nerol	0	67	72	58	75
	Citronellol	0	510	516	322	396
	Geranial	37	213	208	210	209
	Neral	18	123	131	125	122
	Citronellal	0	32	30	28	37
	Geranyl acetate	0	41	33	33	37
	Citronellyl acetate	0	29	32	27	33
	Geranic acid	0	944	954	831	988
	Neric acid	0	1,306	1,834	1,548	1,732
	Citronelic acid	0	241	253	184	196
Other monoterpenes	Rose oxide	0	5	7	5	6
	Myrcene	0	413	363	324	379
	Limonene	8	108	114	82	139
	<i>cis</i> - β -Ocimene	0	123	133	110	127
	<i>trans</i> - β -Ocimene	0	241	223	190	233
Norisoprenes	<i>allo</i> -Ocimene	0	74	69	56	72
	6-Methyl-2-hepten-1-one	171	66	56	56	53
	Geranyl acetone	79	42	34	32	37
	<i>cis</i> -Pseudoionone	12	11	10	12	12
	<i>trans</i> -Pseudoionone	24	22	20	21	21
	Farnesyl acetone	422	416	471	273	348
	Dihydroactinodioliide	29	37	41	33	37
Phenolic derivatives	β -cyclocitral	9	12	13	12	12
	β -ionone	38	47	49	49	46
	2-Isobutylthiazole	217	165	117	107	252
	Benzyl alcohol	44	48	27	60	37
	<i>o</i> -Guaiaicol	115	88	153	129	122
	Methyl salicylate	551	474	388	636	455
	4-Vinylphenol	213	350	263	308	169
	2-Methoxy-4-vinylphenol	145	174	158	175	79
Carotenoids	Eugenol	259	250	73	391	103
	Concentration (μ g/g FW)					
	Phytoene	5.0	0.5	0.8	1.6	1.0
	Lycopene	97.0	48.0	48.0	47.0	53.0
	β -carotene	5.3	5.0	6.3	4.1	5.8

Values represent the means of two independent determinations of ripe fruits (10 d after 'breaker' stage). Similar compositions were obtained for ten more independent lines (data not shown).

a key precursor of the isomeric monoterpene aldehydes geranial and neral (both having a typical lemon aroma), and of nerol, citronellol and geraniol and citronellol acetate esters (all having a rose-like aroma)^{11–13} (**Fig. 1a**).

The expression of *GES* in ripening tomatoes caused marked changes in the volatiles *vis-à-vis* control fruit (**Table 1**). In the transgenic fruits, the concentrations of the monoterpenes geraniol, nerol, citronellol and citronelic acid ranged between 58 and 516 ng/g fresh weight (FW); these compounds were absent in control fruit. Geranic and neric acids, present in the transgenic lines (\sim 900 ng/g FW and \sim 1,500 ng/g FW, on average, respectively), were similarly lacking in control fruits. The levels of neral plus geranial were sixfold higher in *GES*-expressing fruits than in control fruit (355 versus 55 ng/g FW; **Table 1**). Ripe tomatoes do not have endogenous *GES* activity and the low levels of geranial and neral present in controls are probably due to carotenoid degradation¹⁴. Citronellal, citronellyl acetate, geranyl acetate and rose oxide were detected in low concentrations in the transgenic fruit (5–37 ng/g FW), but were absent in the control fruit.

All the above compounds share a common chemical backbone and are probably derived from geraniol (**Fig. 1a**).

A tenfold increase in monoterpene synthase activity and a dramatic induction of *GES* transcripts were detected in transgenic fruit relative to controls (data not shown). As *GES* exclusively catalyzes the geraniol synthesis from GDP when overexpressed in *Escherichia coli*⁹, accumulation of the novel geraniol derivatives likely results from the action of endogenous enzymes that accept geraniol or its derivatives as substrates. As demonstrated¹³, tomato cell-free extracts display high levels of alcohol dehydrogenase activity and produce geranial and neral upon the addition of geraniol and NAD⁺ (data not shown). Citronellol was probably produced by the action of an as-yet unidentified reductase activity similar to those found in geranium and rose¹². Citronellal was probably formed by endogenous alcohol dehydrogenase activity (**Fig. 1a**). Geranyl acetate and citronellyl acetate are likely produced by the action of an endogenous acetyl-CoA:alcohol acetyl transferase activity (data not shown).

Previous attempts to divert the plastidial terpenoid pathway in tomato fruits for aroma modification relied on the use of the tomato fruit-specific *E8* promoter coupled to the *Clarkia breweri* S-linalool synthase (*LIS*) gene¹⁵. The amount of linalool and its derivatives (400–800 ng/g FW) that accumulated in those tomatoes was lower than that in the *GES* transgenic tomatoes described here, which contained about 3,500 ng/g FW geraniol and its derivatives (Table 1). Although the *E8-LIS* transgenic fruits displayed slight alterations in their aroma, the accumulation in the *PG-GES* transgenic fruits of much higher levels of geraniol and its derivatives (some of them possessing typical easily recognizable and strong odors) caused profound changes in the flavor of the fruits (see below).

The transgenic fruits failed to develop the deep red color of the control fruits (Fig. 1b), owing to a 50% drop in the level of lycopene but not of β -carotene (Table 1). Interestingly, phytoene levels were also affected (70–90% decrease). We conclude from these results that the high levels of *GES* expression caused a marked depletion of the GDP pool available for lycopene and phytoene biosynthesis. Such a phenotype was not displayed by *LIS* transgenic tomatoes, in which lycopene levels remained unaffected¹⁵.

Norisoprene volatiles in tomato are derived from the degradation of carotenoids. The decrease of lycopene and phytoene levels correlated with a concomitant decrease in geranyl acetone and 6-methyl-2-heptene-1-one, corroborating the premise that these norisoprenes are probably derived from the degradation of lycopene and phytoene^{6,14}. Conversely, β -ionone, β -cyclocitral and dihydroactinodiolide, which arise from the degradation of β -carotene, remained unaffected (Table 1).

Transgenesis also led to the unexpected accumulation of other monoterpenes such as limonene, myrcene and *allo*-, *cis*- β - and *trans*- β -ocimene, which were either completely absent or present in low concentrations in control fruits (Table 1). A similar phenomenon was detected in *LIS*-transgenic tomatoes¹⁵. This intricate mechanism for monoterpene regulation in tomato fruit requires further examination.

The increases in levels of key volatiles (Fig. 1c) suggested a marked impact on organoleptic attributes of the fruits. Accordingly, >90% of the untrained panelists reported differences in their perception of the smell (orthonasal route). Most of them (79% of the total panelists) reported a stronger (or special) smell in transgenic fruits than in controls (Fig. 1d), whereas 12% of the panelists noted a stronger smell in control fruit; 9% reported no difference (Fig. 1d). The panelists described novel aromas associated with “perfume,” “rose,” “geranium,” “tomato-like” and “lemongrass” notes, in accordance with the modified volatiles composition of the transgenic fruits (Table 1). Furthermore, the panelists reported significant differences ($P = 1.4 \times 10^{-5}$) in the perception of aroma in taste trials (retronasal route)¹⁶ (Fig. 1e). Among the evaluators, 86% judged the retronasal aroma of the transgenic fruits to be different from that of the control fruit, and 68% indicated that the transgenic samples were more aromatic

(Fig. 1e). More than 60% of the panelists preferred the transgenic flavor, 35% preferred the control tomatoes and only 5% expressed no particular preference (Fig. 1f). Even though the evaluators, at times, ranked the transgenic samples as sweeter than the controls, no significant differences were found in total soluble solids, sugar content, pH or total acids (data not shown).

In conclusion, our data clearly indicate that improvement of fruit aroma and taste by genetic engineering is feasible for tomato and that this approach has potential for modifying the aroma and taste of other carotenoid-accumulating species of agricultural and horticultural importance. Moreover, as volatile terpenoids possess antimicrobial, pesticidal and antifungal activities, such manipulations may also improve shelf life of stored fruits or reduce pesticide use.

Experimental procedures are detailed in **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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