Altered metabolite accumulation in tomato fruits by coexpressing a feedback-insensitive AroG and the PhODO1 MYB-type transcription factor

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Introduction

Plant secondary metabolites act as colour pigments, nutrients, components of flavour, protectants against ultraviolet (UV) light, signalling molecules, as well as cell wall components in plants (Liu et al., 2015; Maeda and Dudareva, 2012; Tzin and Galili, 2010). As the best characterized precursors for synthesis of secondary metabolites, the aromatic amino acids (AAAs), including phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr), are derived from chorismate, the final metabolite of the shikimate pathway followed by the aromatic amino acids metabolic pathways. In fact, more than 30% of the fixed carbon in vascular plants is directed towards synthesis of aromatic amino acids via the shikimate and aromatic amino acid biosynthesis pathways (Maeda and Dudareva, 2012; Tohge et al., 2013; Tzin and Galili, 2010). So far, numerous genes encoding regulators of AAA biosynthesis and downstream secondary metabolic pathways have been identified in various plant species. Among them are members of the MYB family of transcription factors.

A large number of MYB regulators that participate in AAA biosynthesis have been characterized to date (Liu et al., 2015). For example, expression of the first gene in the shikimate pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS; EC 2.5.1.54), is regulated by the MYB transcription factor ATR1/MYB34 in Arabidopsis (Bender and Fink, 1998). Knockdown of MYB8 in Nicotiana attenuata significantly reduces expression of all seven shikimate pathway genes, eventually leading to complete elimination of phenylpropanoid–polyamine conjugates (Kaur et al., 2010). The role of MYB transcription factors in controlling the Phe-derived pathways is apparent, including in the Solanaceae family. For example, SIMYB12 is involved in regulating biosynthesis of phenylpropanoids, particularly flavonoids, in tomato fruit (Adato et al., 2009; Luo et al., 2008). Production of the Phe-derived phenylpropanoids/benzzenoids in petunia flowers is controlled by a complex that contains a C2H2-type zinc finger DNA-binding protein, EPF1, and two R2R3-type MYB transcription factors, ODORANTI (PhODO1) and EMISSION OF BENZENOID II (EOBII) (Spitzer-Rimon et al., 2010; Van Moerkercke et al., 2009; Luo et al., 2005). The R2R3-MYB-like gene, EOBII, has been implicated in the direct regulation of PhODO1 (Spitzer-Rimon et al., 2012). Notably, knockdown of PhODO1 in petunia results in higher accumulation of the EOBII transcript, suggesting a complex feedback loop between these regulatory factors. Interestingly, ectopic expression of PhODO1 in tomato fruits resulted in higher levels of a specific subset of phenylpropanoid compounds, but no changes were observed in the levels of Phe-derived flavour volatiles (Dal Cin et al., 2011). This result suggests that, in contrast to petunia, PhODO1 does not interact with the promoters of genes responsible for Phe-derived volatiles when overexpressed in tomato fruit.

To date, different strategies to promote the production of Phe-derived secondary metabolites have been employed (e.g. Zhang et al., 2015). One important aspect of achieving higher phenylpropanoid content is identification of bottlenecks in the
conversion of primary metabolites into secondary metabolites. Examples of enzymes associated with the synthesis of Phe-derived volatiles include the aromatic L-amino acid decarboxylases (Gutensohn et al., 2011; Tieman et al., 2006a), phenylacetaldehyde synthase (Kaminaga et al., 2006) and isoeugenol synthase 1 (Dexter et al., 2007). Our previous studies also indicated that overexpression of the bacterial AroG gene, encoding DAHPS in plants, alleviates a bottleneck in the conversion of primary to secondary metabolites, resulting in enhanced levels of multiple secondary metabolites, including aroma volatiles (Oliva et al., 2015; Tzin et al., 2012, 2013).

In this study, we were interested in understanding the relationships between volatile and nonvolatile phenylpropanoid metabolites summarized in Figure 1. Specifically, our goals were as follows: (i) investigating the effects of the combination of PhODO1 and AroG in regulating the production of volatile and nonvolatile Phe-derived secondary metabolites via metabolic engineering; (ii) producing tomato with higher flavour metabolites. Our results reveal that coexpression of PhODO1 and AroG redirected the flow of phenylpropanoids to certain secondary metabolites in tomato fruit in a distinctly different way than either single transgene. While coexpression did result in lower phenylpropanoid flavour volatiles than AroG alone, they were still significantly higher than in wild-type or PhODO1 plants. Coexpressing plants rerouted Phe into multiple nonvolatile phenylpropanoids with known antioxidant properties. These results shed light on the multiple regulatory roles of ODO1 and AroG in the conversion of primary to Phe-derived secondary metabolism. They also provide a basis for more precise metabolic engineering of tomato to produce desired target secondary metabolite products.

**Results**

**Generation of tomato lines overexpressing the AroG and ODO1 proteins**

As AroG and ODO1 have each been shown to impact secondary metabolism in different ways in tomato fruit (Dal Cin et al., 2011; Tzin et al., 2013), we were interested in coexpressing these two genes to examine their effects on the levels of both volatile and nonvolatile secondary metabolites in tomato fruits (Figure 1). To this end, the ODO1-overexpressing tomato line 8117 was selected for these studies as it exhibited the highest expression of all the transgenic lines (Dal Cin et al., 2011). Subsequently, three independent AroG-overexpressing tomato lines were individually crossed with the ODO1-8117 line to generate transgenic lines whose fruits coexpress the AroG and PhODO1 transgenes (hereafter referred to as AO). Quantitative real-time PCR (qRT-PCR) analysis revealed that the transcript level of PhODO1 was similar in the ODO1 transgenic plant and most of the AO lines (Figure 2a), while the expression of AroG was attenuated in most of the AO lines compared to their corresponding AroG parent lines (Figure 2b).

As expression of AroG in the AO69 (AroG69 × PhODO1) lines was only slightly reduced relative to the corresponding AroG transgenic line, we selected AO69 for subsequent studies. Consistent with previous observations (Dal Cin et al., 2011; Tzin

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**Figure 1** Overview of metabolic pathways altered in AroG and ODO1 expression. Metabolites with asterisks are precursors of multiple glycosylated forms.
et al., 2013), phenotypic analysis showed that overexpressing ODO1 retarded the maturation and/or ripening of fruits while overexpression of AroG had no influence on ripening. Notably, the AO fruit displayed a phenotype similar to that of ODO1 transgenic line (Figure 2c).

Metabolic changes in wild-type, ODO1, AroG and AO fruits

To explore the effect of the combined transgenes in AO on the metabolism of the fruit, two types of tissues (peel and flesh) from the red ripe stage were separately collected for metabolite analysis using high-resolution liquid chromatography/mass spectrometry (LC-MS). To obtain a general view of the differences in metabolite profiles among different genotypes, a principal component analysis (PCA) was conducted with the LC-MS dataset. PCA of the extracts from both peel and flesh tissue showed that the metabolic profiles of wild type (cv. M82), AroG, ODO1 and AO were entirely separated from each other, indicating four distinct metabolic outcomes (Figure 3a,b). To further test whether the levels of metabolites related to the shikimate pathway were altered in AO fruit, we examined the levels of amino acids in the various genotypes. The levels of Phe were significantly reduced in ODO1, elevated in AO and very highly elevated in AroG fruits (Figure 3c). Tyr was significantly elevated in all three transgenic genotypes relative to M82 (Figure 3d). Trp levels were not significantly different from M82 in any of the transgenic genotypes (Figure 3e). Together, these results indicate that AroG has a significant positive effect on synthesis of both Phe and Tyr while minimally impacting Trp levels. ODO1, on the other hand, has an overall negative effect on Phe content while positively impacting Tyr content. Again, Trp accumulation was not impacted. The observed Phe depletion can be observed in both ODO1 fruits relative to M82 and AO plants relative to AroG. As expression of most of the genes in the

![Figure 2](image1.png)  
**Figure 2** Characterization of tomato lines coexpressing ODO1 and AroG. Transcript levels of PhODO1 (a) and AroG (b) in fruits of the three genotypes relative to M82 (±SE). *P < 0.05, **P < 0.01. ODO1, line 8117; AroG69/71/74, three independent lines of AroG; AO69, AroG69 × PhOD11-8117 F1-1; AO71, AroG71 × OD11-8117 F1-9; AO74, AroG74 × OD11-8117 F1-5. (c) Fruit ripening phenotypes of AroG and OD1 coexpression lines. M82, control; ODO1, line 8117 expressing ODO1; AroG, line expressing AroG; AO F1, F1 generation of lines 8117 and AroG. Bar = 1 cm.

![Figure 3](image2.png)  
**Figure 3** Metabolic profiles of nonvolatile phenylpropanoids in four tomato genotypes. PCA analyses of the metabolites identified in peel (a) and flesh (b) tissues. Levels of Phe (c), Tyr (d) and Trp (e) in peel and pericarp tissue of ODO1 (PhOD11-8117), AroG (AroG69) and AO (AO69 F1-1) lines are presented as fold change relative to M82 (n = 4). Letters represent significant difference among the four genotypes using ANOVA (P < 0.05) followed by a Newman–Keuls test (P < 0.05).
shikimate and aromatic amino acid biosynthesis pathways is elevated in ODO1 fruits (Dal Cin et al., 2011), the overall decrease in Phe content associated with the presence of ODO1 is likely due to coordinate up-regulation of downstream phenylpropanoid synthesis genes that substantially increase the levels of multiple phenylpropanoid compounds (described below), thus depleting the content of Phe in the fruit. Consistent with that interpretation, Tyr levels were positively impacted by the presence of the ODO1 transgene. It is interesting to note that despite the increased metabolic activity associated with the shikimate pathway, neither transgene altered Trp levels. We previously showed that ODO1 overexpression resulted in increased transcript accumulation of chorismate mutase but not anthranilate synthase. Unexpectedly, the levels of Leu and Ile were significantly increased in all three transgenic lines relative to M82 (Figure S1).

Coexpression of AroG and ODO1 alters the profiles of nonvolatile phenylpropanoid metabolites in fruit peel and flesh tissues

We were interested in examining the changes in the nonvolatile phenylpropanoids. Therefore, we first investigated the metabolite changes in peels of M82, AroG, ODO1 and AO fruits (Table S1). The levels of eight and 12 metabolites were significantly higher or lower in ODO1 as compared to M82, respectively (Table 1). Notably, the metabolites with lower content mainly consisted of naringenin-derived metabolites. In AroG, there were eight significantly elevated metabolites relative to M82, including multiple coumaric acid-derived metabolites. Only two metabolites, methylbutanol-hexose-pentose and phloretin-trihexose, were significantly reduced in AroG relative to M82 (Table 1). There were substantially more metabolites altered in the AO fruit with the levels of 20 metabolites significantly increased and 11 reduced, relative to M82. Metabolites with significant increases included ferulic acid-, kaempferol- and quercetin-derived compounds. Among them, the levels of eight metabolites were significantly increased in AO as compared to those in the wild-type, ODO1 or the AroG genotypes (Table 1). Interestingly, the pattern of metabolites with significant variations in ODO1 was similar to that of AO. For example, there were increases in ferulic acid- and kaempferol-derived metabolites in ODO1 and AO as compared to M82, whereas naringenin- and quercetin-derived metabolites were significantly lower in ODO1 and AO. These results suggest that ODO1 specifically alters expression of genes whose products direct biosynthesis of these secondary metabolites. In addition, a few metabolites were different from M82 in AroG and AO but not ODO1. Thus, the AO metabolite profile is a combination of the effects of both transgenes.

A total of 40 phenylpropanoid metabolites were putatively identified in the flesh tissues of the four genotypes (Table S1). Of these metabolites, the levels of 15 were significantly different in one or more of the transgenic lines (Table 2). Our results indicate that three metabolites were elevated and one was reduced in ODO1 as compared to M82, whereas only one metabolite in AroG was significantly elevated (Table 2). However, 14 of the 15 metabolites were elevated in AO as compared to M82. These metabolites included caffeic acid-, coumaric acid- and ferulate-derived metabolites. These results indicate that combination of ODO1 and AroG significantly alters metabolite flow into Phenylpropanoid metabolites in tomato type, ODO1 or the AroG genotypes (Table 1). Interestingly, the metabolites with lower content mainly consisted of naringenin-derived metabolites. In AroG, there were eight significantly elevated metabolites relative to M82, including multiple coumaric acid-derived metabolites. Only two metabolites, methylbutanol-hexose-pentose and phloretin-trihexose, were significantly reduced in AroG relative to M82 (Table 1). There were substantially more metabolites altered in the AO fruit with the levels of 20 metabolites significantly increased and 11 reduced, relative to M82. Metabolites with significant increases included ferulic acid-, kaempferol- and quercetin-derived compounds. Among them, the levels of eight metabolites were significantly increased in AO as compared to those in the wild-type, ODO1 or the AroG genotypes (Table 1). Interestingly, the pattern of metabolites with significant variations in ODO1 was similar to that of AO. For example, there were increases in ferulic acid- and kaempferol-derived metabolites in ODO1 and AO as compared to M82, whereas naringenin- and quercetin-derived metabolites were significantly lower in ODO1 and AO. These results suggest that ODO1 specifically alters expression of genes whose products direct biosynthesis of these secondary metabolites. In addition, a few metabolites were different from M82 in AroG and AO but not ODO1. Thus, the AO metabolite profile is a combination of the effects of both transgenes.

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appropriate transgene. The second tool is a transgene encoding PhODO1, a petunia MYB transcription factor. Overexpression of this gene in tobacco results in higher expression of genes encoding most of the steps for synthesis of Phe and Tyr as well as multiple enzymes responsible for synthesis of many nonvolatile phenylpropanoids (Dal Cin et al., 2011). Notably, ODO1 overexpression in tomato causes higher expression of multiple isoforms of PHENYLALANINE AMMONIA LYSASE (PAL), the first step in synthesis of most phenylpropanoids. Here, we have combined expression of the mutant E. coli DAHPS and ODO1 in a single...
Table 2 Differentially expressed metabolites in the flesh of AO fruits

| Metabolites                        | WT          | ODO1        | AroG        | AO          |
|-----------------------------------|-------------|-------------|-------------|-------------|
| Caffeic acid hexose isomer 1      | 1 ± 0.36 (a)| 0.43 ± 0.09 (a)| 17.81 ± 11.61 (ab)| 27.56 ± 9.07 (b) |
| Caffeic acid hexose isomer 2      | 1 ± 0.09 (a)| 1.17 ± 0.68 (a)| 5.24 ± 4.06 (ab)| 8.63 ± 2.52 (b) |
| Caffeic acid hexose isomer 3      | 1 ± 0.09 (a)| 1.78 ± 0.44 (a)| 0.73 ± 0.56 (a)| 8.17 ± 0.66 (b) |
| Coumaric acid hexose isomer 1     | 1 ± 0.3 (a) | 0.87 ± 0.37 (a)| 2.1 ± 1.6 (a)  | 8.1 ± 1.66 (b)  |
| Coumaric acid hexose isomer 2     | 1 ± 0.14 (a)| 0.99 ± 0.27 (a)| 1.25 ± 0.88 (a)| 9.08 ± 2.18 (b) |
| Coumaric acid hexose isomer 3     | 1 ± 0.54 (a)| 5.56 ± 1.94 (a)| 4.85 ± 3.03 (a)| 31.06 ± 7.15 (b)|
| Coumaroylquinic acid              | 1 ± 0.3 (ab)| 0.77 ± 0.18 (a)| 1.24 ± 0.94 (ab)| 5.28 ± 1.21 (b) |
| Ferulic acid hexose isomer 1      | 1 ± 0.07 (a)| 0.81 ± 0.26 (a)| 0.85 ± 0.57 (a) | 3.77 ± 2.02 (b) |
| Feruloylquinic acid               | 1 ± 0.25 (a)| 5.26 ± 0.7 (bc)| 2.01 ± 1.46 (ab)| 13.4 ± 2.31 (c) |
| Feruloylquinic acid-O-hexoside    | 1 ± 0.23 (a)| 14.88 ± 7.7 (b)| 0.83 ± 0.43 (a) | 27.66 ± 3.8 (c) |
| Hydrocinnamic acid hexose         | 1 ± 0.25 (a)| 3.77 ± 1.11 (a)| 2.21 ± 1.93 (a) | 32.2 ± 2.86 (b) |
| Methylbutanol-hexose-pentose      | 1 ± 0.45 (a)| 10.9 ± 1.53 (b)| 0.18 ± 0.15 (a) | 4.23 ± 1.48 (c) |
| Naringenin chalcone               | 1 ± 0.31 (b)| 0.06 ± 0.01 (a) | 0.44 ± 0.32 (ab) | 0.07 ± 0.04 (a) |
| Tyrosine                          | 1 ± 0.1 (a) | 0.82 ± 0.14 (a)| 7.27 ± 3.48 (b)  | 21.3 ± 2.79 (c) |

Numbers (n = 3; mean ± SE) are the fold change as compared to wild type (M82), and the numbers in bold indicate significant up-regulation of the corresponding metabolites in AO, as compared to that in the other three genotypes. Boxes in yellow or green represent the up-regulation or down-regulation of the level of metabolite in corresponding genotype as compared to that in wild type. The letters in parentheses represent significant difference among the four genotypes by using ANOVA (P < 0.05) and the Tukey test for corrections for multiple comparisons (P < 0.05).

Figure 4 Effect of ODO1, AroG and the combination on benzenoid-phenylpropanoid volatile content. Levels (±SE) of phenylpropanoid volatiles in fruits of ODO1 (PhODO1-8117), AroG (AroG69) and AO (AO69 F1-1) lines. Letters represent significant difference among the four genotypes using ANOVA (P < 0.05) followed by a Newman–Keuls test (P < 0.05). Results are presented as fold change of each volatile relative to M82 (n = 3).
plant and observed the effects of that overexpression on a broad array of volatile and nonvolatile phenylpropanoid metabolites in tomato fruits. The overall effects on metabolite content are summarized in Figure 5.

AroG expression alone has a large, positive effect on Phe content (Figure 3) but a minimal effect on nonvolatile phenylpropanoids in the fruit flesh; only one of 40 measured compounds was significantly higher (Table 2). Several additional compounds were significantly increased in fruit peel (Table 1), but overall, the large increase in Phe did not have a major impact on chemicals downstream of PAL. This result suggests that PAL expression and not the amount of available Phe is the primary determinant of commitment to nonvolatile phenylpropanoid catabolism. In the absence of increased PAL expression, Phe content in AroG plants increases without a major impact on these phenylpropanoids. Some part of that increased available Phe does, however, get shunted into phenylpropanoid volatile synthesis (Figure 4). The first committed step to aromatic volatile synthesis is catalysed by a family of aromatic amino acid decarboxylases (AADC) (Tieman et al., 2006a). Although expression of AADC genes does impact volatile content, the present results indicate that availability of Phe can influence the output of this pathway.

Overexpression of ODO1 alone actually led to a decrease in the content of Phe in the fruit. This decrease was accompanied by a major redistribution of multiple phenylpropanoids. Many exhibited significant increases, particularly those derived from ferulic acid, while others such as flavonoids were lower. We have previously reported increased fluxes of 7- to 100-fold into some phenylpropanoids in ODO1 overexpressing tomato fruits (Dal Cin et al., 2011) and these results are consistent with those earlier findings. That increased metabolic demand results in a reduction in the amount of free Phe and that smaller available Phe, in turn, leads to reduced levels of the phenylpropanoid volatiles (Figure 4).

The chemical contents of AO fruits exhibit properties of both ODO1 and AroG expression. However, the dominant effect of ODO1 expression is readily apparent. Fruit peel and flesh contain very high levels of nonvolatile phenylpropanoids (Tables 1 and 2). In many instances, the levels are substantially higher than those observed with either single transgene. For example, the levels of multiple caffeic acid and coumaric acid sugar conjugates are much higher in AO plant flesh. This significantly higher phenylpropanoid content is presumably due to the combination of high expression of genes encoding PAL and other downstream metabolic enzymes with much higher availability of Phe catalysed.

Figure 5 Changes of the primary and secondary metabolites in the four tomato genotypes. Green boxes indicate reduced levels, while red boxes indicate increased levels of the given metabolites. Volatile metabolites are highlighted in orange. Boxes with black dashed lines represent no corresponding metabolites detected in the flesh tissue; Boxes with orange outlines represent volatile metabolites detected from both peel and flesh tissue.
by the presence of AroG. That increased demand is evidenced by the lower level of Phe in AO relative to AroG fruit. That reduction of available Phe also impacts the ability to synthesize phenylpropanoid volatiles. Phe levels are intermediate between levels observed in M82 and AroG fruits, as are the levels of these volatiles. Indeed, only 2-phenylethanol remains significantly higher than M82 in the AO fruits. Taken together, we conclude that the demand imposed by higher PAL gene expression shunts Phe into nonvolatile phenylpropanoids at the expense of the phenylpropanoid volatiles.

The level of Tyr in the transgenic lines is consistent with the above arguments. Expression of ODO1 in fruits increases expression of most of the genes responsible for Phe and Tyr synthesis (Dal Cin et al., 2011). But the downstream demand for Tyr is far less than for Phe. Thus, ODO1 fruits have significantly elevated Tyr content than do M82 fruits (Figure 3). AroG, by itself, also very significantly increases Tyr content relative to M82. The combination of the two transgenes in AO is synergistic with yet again higher levels of Tyr. Thus, the reduction of Phe in AO fruits relative to AroG is likely due to increased demand for synthesis of nonvolatile phenylpropanoids.

Much attention has been paid to the health promoting activities associated with phenylpropanoids, particularly anthocyanins and flavonols in tomato fruit in particular (Bovy et al., 2002; Butelli et al., 2008; Luo et al., 2008). Synthesis of these compounds is regulated by MYB transcription factors [reviewed in (Luo et al., 2010)]. Thus, fruits with higher levels of the hydroxycinnamic acids, including caffeic, chlorogenic and coumaric acids, are also antioxidants and antimicrobials (Korkina, 2007; Luo et al., 2008; Naoumkina et al., 2010). Thus, fruits with higher levels of the hydroxycinnamic acids are also potentially valuable. While the ODO1 fruits have higher levels of hydroxycinnamic acids, the AO plants have significantly more of both the volatile and nonvolatile phenylpropanoids. They also contain significantly more Phe and Tyr, both essential amino acids. Thus, the AO fruits have the potential to be both more flavourful and contain higher levels of antioxidants. Whether they are perceived as having superior flavour remains to be determined. It would also be of great interest to combine AroG expression with the MYB genes that direct higher flavonol and anthocyanin synthesis such as MYB12 (Luo et al., 2008) and MYB112 (Lotkowska et al., 2015), as we would predict that these combinations would lead to further increases in those chemicals.

Conclusion

Here, we describe plants altered in both soluble and volatile phenylpropanoid composition as a consequence of transgenic expression of a plant MYB transcription factor, a bacterial feedback-insensitive DAHPS (AroG) and a combination of the two. Expression of either single gene or the combination of the two led to three distinct metabolic outcomes. Expression of AroG alone resulted in higher accumulation of volatile and nonvolatile phenylpropanoids. Expression of ODO1 alone resulted in large increases in nonvolatile phenylpropanoids with minimal effect on volatiles, presumably due to the large increase in PAL expression, leading to diversion of Phe into the nonvolatile pathways. Coexpression of both genes resulted in further increases in nonvolatile phenylpropanoids, especially the hydroxycinnamic acids, with some increase in volatile phenylpropanoids. The consequences of these manipulations indicate a way forward for increasing both flavour-associated volatile and antioxidant phenylpropanoids.

Materials and methods

Plant material and growth conditions

Greenhouse-grown plants used in this study were in the M82 background, including the AroG and ODO1 transgenic plants. Each biological repeat was a mixture of five to six individual fruit in the ripe red stage (manually dissected peel and flesh tissues without the gel and seeds). To generate the AroG and ODO1 coexpression lines, homozygous ODO1 lines were crossed with three independent homozygous AroG transgenic lines and the resulting F1 plants were used for this study.

Metabolic profiling using high-resolution LC-MS

Nontargeted metabolic analysis was performed using 100 mg of frozen powder from tomato peel and flesh tissues, extracted in 80% methanol. Samples were analysed using an UPLC/qTOF system (HDMS Synapt; Waters Milford MA, USA), with the UPLC C18 column connected online to a photodiode array detector and then to the MS detector, in MS3 acquisition mode. Sample preparation and injection conditions were performed as previously described (Mintz-Oron et al., 2008). Analysis of the raw LC-MS (UPLC/qTOF-MS) data was performed using the XCMS software from the Bioconductor package (v. 2.1) for the R statistical language (v. 2.6.1) that performs chromatogram alignment, mass signal detection and peak integration (Smith et al., 2006). XCMS was used with the following parameters: fwhm = 10.8, step = 0.05, steps = 4, mzdiff = 0.07, snthresh = 8, max = 1000. Injections of samples in the positive and negative ionization modes were performed in separate injection sets and preprocessing was done for each ionization mode independently. The list of putatively identified compounds (totally 69 metabolites in both peel and flesh), including their exact masses, retention times and the main fragments, is present in Supplemental Table 1. Differential mass ions were determined using a Student’s t-test (JMP software) and 17 metabolites whose levels were significantly different from the control M82 were subsequently identified. Principal component analysis (PCA) was performed with the TMEV4 software (Scholz et al., 2004). A Student’s t-test analysis was performed on metabolites using the JMP software (SAS).

Volatiles Collection and Analysis

Collection of volatile compounds was performed as described previously (Tieman et al., 2006b). In brief, volatiles were collected from chopped ripe fruits (peel and flesh) during a 1-h period. The volatiles were trapped on SuperQ resin and subsequently eluted with methylene chloride using nonyl acetate as an internal control. The samples were separated on a DB-5 column (Agilent, www.agilent.com) and analysed on an Agilent 6890N gas chromatograph equipped with a flame ionization detector. Retention times compared with known standards and identities of volatile peaks were confirmed by gas chromatography mass spectrometry (GC-MS) (Agilent 5975 GC-MS, www.agilent.com). The list of quantified volatile compounds is presented in Supplemental Table 2. The principal component analysis (PCA) plot was performed with MetaboAnalyst 3.0 web tool (Xia et al., 2015).
For multiple comparisons, ANOVA was performed followed by a Newman–Keuls test with SAS V8 (SAS Institute Inc., Cary, NC, USA). The level of significance is indicated in each Figure.

**Amino Acid Purification, Derivatization and GC-MS Analysis**

Amino acid purification, derivatization and GC-MS analysis were performed according to a previous study (Chen et al., 2010). Briefly, tomato pericarp tissue was frozen in liquid nitrogen and ground into fine powder. The powder was mixed with 2.5 mL of 10 mM HCI and 20 µL of 10 mg/mL methane sulphonium as internal standard in a mortar by grinding. Samples were placed in scintillation vials and shaken for 45 min. Sample tubes were centrifuged at 16 000 g for 3 min, supernatants were drawn through SCX SPE columns (Grace Davison Discovery Science) into a vacuum manifold, and amino acids were eluted with 1 mL of 1:1(v/v) 4 M NH4OH: methanol. One hundred µL aliquot of the analyte was transferred to a GC-MS vial insert and derivatized by mixing with 15 µL of pyridine and 15 µL of methyl chloroformate (MCF). To separate the MCF derivatives from the reactive mixture, 300 µL of chloroform and 200 µL of sodium bicarbonate (50 mM) were added and vortexed. The bottom layer was transferred to a GC insert containing crystals of anhydrous sodium sulphate to dry the samples before they were used for GC-MS analysis. The mass spectra of the MCF derivatized amino acids and internal standards were obtained in SIM acquisition mode.

**RNA extraction and quantitative real-time PCR**

Total RNA was prepared from fruit tissue using ISOLATE II RNA Plant Kit (Bioline, Taunton, MA, USA). Genomic DNA contamination was removed by DNase treatment (ROQ RNase-Free DNase Product, Promega, Madison, WI, USA). Quantitative PCR was performed with a StepOnePlus real-time PCR system using SensiFAST™ SYBR Hi-ROX One-Step Kit (Bioline). The oligonucleotides used for quantitative real-time PCR were as follows: AroG: forward 5′-ATCACCCCCACAA TATCTCGC-3′ and reverse 5′-AGGCACCTTATGTCAGCCGC-3′; PHOD1: forward 5′-GAAAACCTTCTAGCACCC-3′ and reverse 5′- TCAAAAC-CAAAGTCATTGATACC-3′.

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**Author contributions**

A. A., H. K. and G. G. designed the research. Q. X. and Z. L. performed the experiments and data analysis assisted by S. M. and I. R. Q. X., Z. L., A. A., H. K. and G. G. wrote the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Leu and Ile content in different genotypes.

Figure S2 Principal component analysis of the volatile metabolites derived from the peel and flesh tissues in the four genotypes.

Figure S3 Effects of ODO1, AroG and the combination on branched-chain amino acid-derived volatiles.

Table S1 List of putative metabolites identified in the peel and flesh of the four genotypes by UPLC/qTOF-MS at negative mode.

Table S2 List of quantified volatile compounds in the four genotypes by Targeted-GC (ng/gFW/h).