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Dually biofortified cisgenic tomatoes with increased flavonoids and branched-chain amino acids content

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Summary

Higher dietary intakes of flavonoids may have a beneficial role in cardiovascular disease prevention. Additionally, supplementation of branched-chain amino acids (BCAAs) in vegan diets can reduce risks associated to their deficiency, particularly in older adults, which can cause loss of skeletal muscle strength and mass. Most plant-derived foods contain only small amounts of BCAAs, and those plants with high levels of flavonoids are not eaten broadly. Here we describe the generation of metabolically engineered cisgenic tomatoes enriched in both flavonoids and BCAAs. In this approach, coding and regulatory DNA elements, all derived from the tomato genome, were combined to obtain a herbicide-resistant version of an acetolactate synthase (mSlALS) gene expressed broadly and a MYB12-like transcription factor (SlMYB12) expressed in a fruit-specific manner. The mSlALS played a dual role, as a selectable marker as well as being key enzyme in BCAA enrichment. The resulting cisgenic tomatoes were highly enriched in Leucine (21-fold compared to wild-type levels), Valine (ninefold) and Isoleucine (threefold) and concomitantly biofortified in several antioxidant flavonoids including kaempferol (64-fold) and quercetin (45-fold). Comprehensive metabolomic and transcriptomic analysis of the biofortified cisgenic tomatoes revealed marked differences to wild type and could serve to evaluate the safety of these biofortified fruits for human consumption.

Introduction

Personalized nutrition aims to improve life quality and prevent disease by customizing diets based on individual characteristics such as age, genetic background, gut flora composition and behavioural habits. As our ability to generate individualized genetic and metabolomic profiles grows, so does the need to expand the assortment of reliable food sources from which important dietary compounds can be obtained. In recent times, the increasing precision offered by some new plant breeding techniques such as gene editing or cisgenesis has contributed to raising public acceptance of plant biotechnology applied to food, as exemplified by the pioneering commercialization of GABA-enriched gene edited tomatoes (Waltz, 2021). At the same time, new breeding techniques open the way to the design of new metabolically customized fruits that combine noncompeting metabolite enrichment strategies, narrowing the gap between precision nutrition and metabolic engineering. In this work, we describe the engineering of tomatoes dually enriched in flavonoids and branched-chain amino acids (BCAAs) using a cisgenic approach.

Flavonoids have a proposed role in the prevention of cardiovascular diseases and colon cancer (Martin *et al.*, 2013). The overexpression of regulatory genes that activate several enzymes of the pathway was reported to lead to the accumulation at high levels of different flavonoid compounds such as anthocyanins, flavonols or both, depending on the specific combination of transcription factors employed (Bovy *et al.*, 2002; Butelli *et al.*, 2008; Luo *et al.*, 2008; Zhang *et al.*, 2015). In particular, transgenic overexpression of Arabidopsis MYB12 (AtMYB12), a master regulator of the phenylpropanoid biosynthetic pathway, led to a substantial increase in flavonol levels in fruits (Luo *et al.*, 2008).

There are increasing concerns about the effect that strictly vegetarian diets could have in the development of muscle related conditions, especially in older adults. Low body mass index (BMI) and reduced muscle mass have been associated with the increased risk of hip fracture observed in women following vegetarian diets (Webster *et al.*, 2022). Sarcopenia, a condition characterized by loss of muscle mass in older adults, is also a condition of concern associated with diets with low animal protein intake (Beaudart *et al.*, 2017; Huang *et al.*, 2016). BCAAs, valine (Val), leucine (Leu) and isoleucine (Ile), account for 14%–18% of muscle protein total amino acids and they are considered critical regulators of anabolism in skeletal muscle tissues (Brestenský *et al.*, 2015). Supplementation with these essential

amino acids abundant in animal proteins is, alongside with resistance training, a standard treatment for sarcopenia (McKendry *et al*., 2020). Indeed, a lucrative market of BCAA supplements has grown in athletics due to the claims that relate these supplements with an increase in BMI (Kårlund *et al*., 2019). A more desirable solution to dietary supplements would consist in enriching fruits and vegetables in key compounds whose absence could bring long-term negative effects in vegetarian diets. In this regard, elevating BCAA content in fruit and vegetables could reduce problems associated with the highly recommended reduction in the intake of animal-based food in older adults, especially for individuals opting for a strict vegan diet (Domić *et al*., 2022; Reid-McCann *et al*., 2022). An unexplored strategy to increase BCAAs in plant tissues consists of overexpression of the acetolactate synthase (ALS), an enzyme that catalyses the first step of BCAA biosynthesis, in particular the conversion of pyruvate into 2-acetolactate to Val and Leu and the pyruvate conversion into 2-aceto-2-hydroxybutyrate, a precursor of Ile (see Figure 2).

Cisgenesis is a new plant breeding technique (NPBT), which stands astride classical breeding and transgenesis (Espinoza *et al*., 2013). Cisgenesis makes only use of the genetic pool from a plant species itself or cross-compatible ones for engineering crops with new agronomic traits. By avoiding the introduction of alien DNA in the crop's genome, cisgenic approaches aim to reduce the biosafety concerns associated to transgenesis. Cisgenesis requires the use of compatible selection procedures. Some selectable markers have been derived from plants and those conferring resistance to herbicides can be adapted to cisgenic approaches (Liu *et al*., 2013; Sundar and Sakthivel, 2008; Tian *et al*., 2015; Yu *et al*., 2015), including the use of mutated versions of the above mentioned ALS enzyme (Okuzaki *et al*., 2007; Shimizu *et al*., 2008; Yao *et al*., 2013). ALS inhibition by herbicides results in a reduction of BCAAs in the plant and ultimately in plant death (Binder, 2010). Different naturally occurring amino acid substitutions have been reported to confer tolerance to different ALS-inhibiting herbicides which block the entrance of pyruvate to the active site of the enzyme (Zhou *et al*., 2007). One of them, the Pro-197-Ser mutation (in the ALS of *Arabidopsis thaliana*), results in sulphonylurea resistance (Haughn *et al*., 1988). Pro-197 is located at an α-helix within the substrate access channel and is a relevant amino acid for herbicide binding. Thus, the access of the substrate to the catalytic site is impeded when sulphonylureas are present (Zhou *et al*., 2007), being its entrance reverted with a Pro-197-Ser mutation. Selection markers based on this mutated ALS have been developed for crops such as apple (Yao *et al*., 2013) and tobacco (Haughn *et al*., 1988), but an equivalent strategy remains unavailable for tomato.

We show here that the generation of tomatoes dually biofortified in flavonoids and BCAAs can be achieved by combining the constitutive expression of a mutated version of a *Solanum lycopersicum* ALS (mSlALS) gene and the previously described tomato MYB12 gene (SlMyb12) (Ballester *et al*., 2010) driven by a fruit-specific promoter. In this approach, ALS played a dual role, both as cisgenic selectable marker and as key factor for BCAA enrichment. Metabolic analyses demonstrate that cisgenic fruits were enriched in flavonols with significant accumulation of rutin, quercetin and kaempferol in the fruit flesh. Additionally, our data show that overexpression of mSlALS results not only on herbicide resistance but also in increased content of BCAAs and BCAA-derived volatiles. Finally, we used transcriptomics and metabolomics to provide an unbiased picture of the effects in the fruit of the simultaneous expression of SlMYB12 and SlALS.

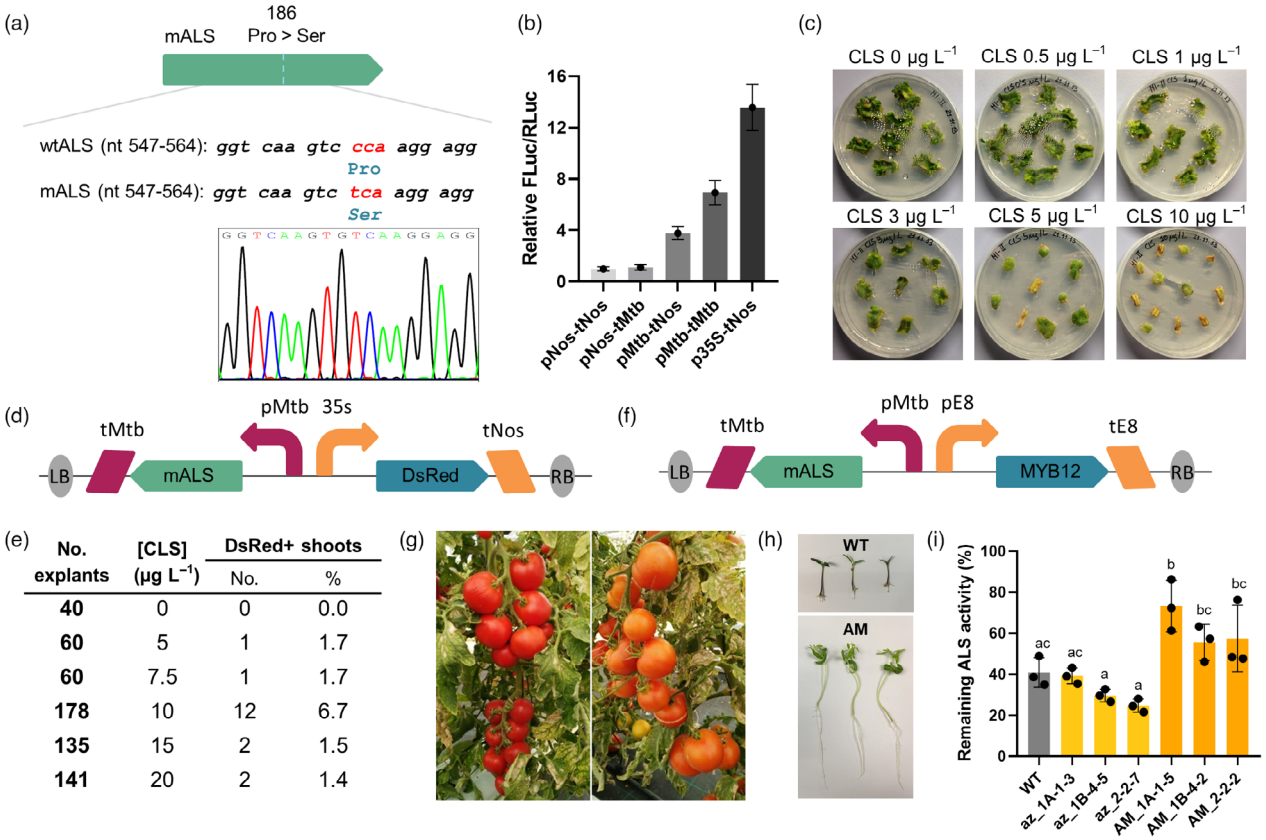
Results

Design of a new strategy for tomato cisgenic biofortification

An all-tomato-based selectable marker requires an endogenous gene expressed under tomato regulatory sequences. We aimed to design a cisgenic selectable marker based on ALS. A BLAST search with the AtALS amino acid sequence resulted in the identification of the three putative ALS homologues in tomato previously reported by Gao *et al*. (2014), ALS1 (Solyc03g044330.1), ALS2 (Solyc07g061940.2) and ALS3 (Solyc06g059889.2). Two of them, ALS1 and ALS2, have the highest amino acid sequence similarity with AtALS. We arbitrarily selected ALS1 to set up a cisgenic selection marker for tomato transformation. A single nucleotide change (C to T at position 556), resulting in a proline to serine mutation at amino acid 186 (position 197 in reference to AtALS) was introduced to create a sulphonylureas resistant version of SlALS (mSlALS, Figure 1a). In parallel, tomato promoter regions conferring high and constitutive expression were isolated from genes showing high and broadly distributed expression in the Tomato Functional Genomics Database (http://ted.bti.cornell.edu/). Three top candidate genes (Solyc01g099770, Solyc06g007510 and Solyc09g010800) were selected, and their putative 5′ regulatory regions (comprising the annotated 5′ UTRs plus a 2 kb fragment upstream of the transcriptional start site (TSS)) and 3′ regulatory regions (comprising the 3′ UTRs plus 1 kb fragment downstream the transcriptional termination site) were isolated and tested transiently in *Nicotiana benthamiana* using a Firefly luciferase (FLuc) assay as previously described by Vazquez-Vilar *et al*. (2017). Among the different assayed promoters, the metallothionein-like protein type 2B (Mtb, Solyc09g010800) promoter, in combination with its own terminator, was shown to confer the highest relative expression levels, 6.94 ± 0.95 relative promoter units (RPUs, relative to nopaline synthase promoter) (Figure 1b, Sarrion-Perdigones *et al*., 2013). The transcriptional activity conferred by the Mtb promoter and terminator regions in the assayed conditions was approximately half of the activity conferred by the strong CaMV35S promoter when combined with the nopaline synthase (nos) terminator. Based on these data, we assembled the mSlALS CDS with the Solyc09g010800 promoter and terminator regions generating the transcriptional unit (TU) pMtb::mSlALS::tMtb, ready to be used as a cisgenic selectable marker.

Next, we established a protocol for tomato transformation with the new marker. First, we tested shoot regeneration of untransformed *S. lycopersicum* cv. Moneymaker cotyledon explants at different chlorsulfuron (CLS) concentrations (Figure 1c). Based on these results, we carried out the optimization of the new cisgenic marker for Agrobacterium-mediated transformation using a linked DsRed fluorescent reporter construct (Figure 1d) and testing a range of CLS concentrations in the regeneration media. The number of transformed explants was calculated based on their DsRed fluorescence. The optimal CLS concentration was set at 10 μg L^{-1}, resulting in a transformation efficiency of 6.7% (Figure 1e).

Finally, to obtain mature fruits cisgenically biofortified in flavonoids, we produced a construct where the expression of



No. explants	[CLS] (µg L^{-1})	DsRed+ shoots	
		No.	%
40	0	0	0.0
60	5	1	1.7
60	7.5	1	1.7
178	10	12	6.7
135	15	2	1.5
141	20	2	1.4

Figure 1 Overexpression of a mutated version of the acetolactate synthase gene confers chlorsulfuron resistance in tomato. (a) Schematic representation and confirmatory chromatogram of the nucleotidic mutation introduced in the ALS that results on a Pro > Ser amino acid change at position 186. (b) Relative Firefly Luciferase (FLuc)/Renilla Luciferase (RLuc) activity conferred by different promoter and terminator combinations (Sarrion-Perdigones *et al.*, 2013). (c) Untransformed cotyledon explants maintained for 5 weeks on regeneration medium supplemented with different chlorsulfuron concentrations. (d) Genetic construct used for optimization of the tomato transformation protocol with the mALS selection marker. (e) Number of transgenic shoots recovered with different CLS concentrations in a transformation carried out with the genetic construct depicted in (d). (f) Schematic representation of the genetic construct used for the generation of the ALS-MYB (AM) lines. (g) Photograph of WT (left) and T0 AM (right) ripened fruits. (h) 10-day-old seedlings of WT and cisgenic AM lines grown in MS supplemented with 50 µg L^{-1} chlorsulfuron. (i) Calculated remaining ALS activity in leaf extracts of three independent T2 AM lines and their corresponding azygous lines in the absence and presence of 50 µg L^{-1} chlorsulfuron. Error bars represent SD of independent biological replicates (n = 3). Statistical analyses were performed using one-way ANOVA (Tukey's multiple comparisons test, P-Value ≤0.05). Variables within the same statistical groups are marked with the same letters.

an endogenous MYB12 homologue (SlMYB12, coding sequence of Solyc01g079620.3) was driven by the ethylene-responsive, fruit-specific regulatory regions of the tomato E8 gene (Solyc09g089580.3.1). The assembly of this new TU next to the cisgenic ALS marker resulted in the genetic module tMtb::mSlALS::pMtb-pE8::SlMYB12::tE8 (Figure 1f). Eleven primary transformants were obtained after transformation with this genetic construct from 200 explants (5.5% transformation efficiency). In this T0 generation, some transient differences in fruit colour during early ripening were observed, perhaps attributable to accumulation of naringenin; however, the differences were rapidly masked by subsequent accumulation of red lycopene (Figure 1g). No other obvious phenotypic changes were observed. T1 seeds of three of the lines, hereafter referred to as AM lines, were germinated in MS medium supplemented with 10 µg L^{-1} CLS. Only plants carrying the T-DNA grew in these conditions, confirming the stability of the cisgenic marker (Figure 1h). All three AM lines showed a segregation corresponding to the insertion of a single copy of the T-DNA (data not shown). To further corroborate the CLS resistance observed in seedlings, we determined the ALS enzymatic activity in leaves of AM and wild-type plants. Azygous lines from which the T-DNA had been segregated were used as additional controls. As shown in Figure S1, all AM, azygous and WT plants showed detectable ALS activity, but only AM samples partially retained ALS activity when incubated in the presence of CLS (Figure 1h). The partial loss of ALS activity in cisgenic plants in the presence of the herbicide can be explained by the presence of two additional wild-type ALS gene copies in the genome which are susceptible to CLS. Six T1 plants from each AM line were grown to maturity and self-pollinated to the next generation. All experiments described below were carried out in plants of the T2 and subsequent generations.

AM cisgenic tomatoes show an enrichment of BCAA metabolism in fruit

The expression levels of both cisgenic (cALS) and endogenous (eALS) transcripts were confirmed using a RT-qPCR analysis with

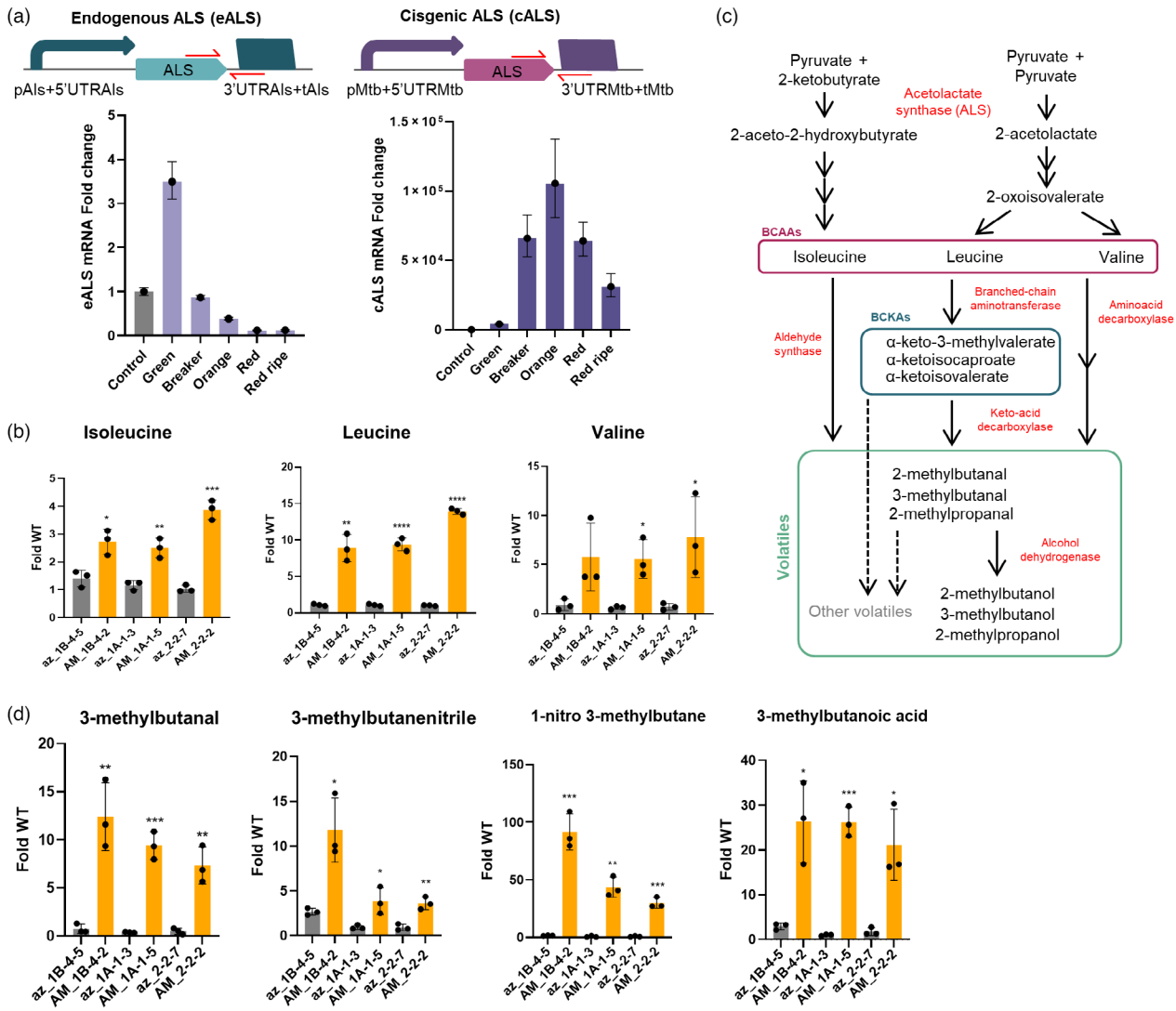


Figure 2 Overexpression of the acetolactate synthase gene results in increased BCAAs and branched chain volatiles in tomato fruits. (a) Primer design for differential detection of the endogenous and the cisgenic *Solanum lycopersicum* ALS mRNA (top) and mRNA fold change of endogenous and cisgenic ALS (bottom). Fruits of a T3 2-2-2 homozygous plant were harvested at different ripening stages. The control sample is a fruit of an azygous line harvested at breaker. Error bars represent standard deviation of technical replicates (n = 3). (b) Fold change on the BCAAs content of red ripe tomato fruits of three independent T2 AM lines compared to their corresponding azygous lines. (c) Schematic representation of the BCAAs biosynthetic pathway and proposed pathways for branched chain volatiles synthesis. (d) Fold change on the branched chain volatiles content of red ripe tomato fruits of three independent T2 AM lines compared to their corresponding azygous lines determined using GC–MS. Asterisks indicate values that are significantly different *(P < 0.05), ** (P < 0.01), ***(P < 0.001) from their corresponding azygous lines (Student's t-test). Error bars represent SD of independent biological replicates (n = 3).

primers specifically designed to differentiate between the two forms (see Figure S2). cALS transcript showed strong over-accumulation in fruit with a 25-fold increment from green to orange stages (Figure 2a), indicating a ripening-associated activation of Mtb promoter that, to our knowledge, had not been shown before. These data led us to investigate the extent to which the high levels of ALS transcripts in the fruit flesh would affect the fruit composition, and in particular, the amount of BCAAs and their derivatives. We found much higher amounts of leucine, isoleucine and valine in the three lines under analysis (Figure 2b). For AM_2-2-2, increments were 14-, 3.8- and 7.8-fold for Leu, Ile and Val, respectively, with respect to WT fruits. Absolute contents of all three BCAAs in fruits of line AM_2-2-2 are shown in Table 1. As can be observed, the accumulation of free leucine reached up to 350 mg kg^{-1} FW (estimated 7.0 g kg^{-1} dry weight), whereas more modest accumulations of isoleucine (45 mg kg^{-1} FW, estimated 0.9 g kg^{-1} DW) and valine (140 mg kg^{-1} FW, estimated 2.8 g kg^{-1} DW) were measured. To discard a possible effect of MYB12 in the changes observed in BCAAs in the fruit, we studied levels of Leu, Ile and Val also in the leaves of the three lines. As expected, BCAAs were found to overaccumulate also in the leaves of cisgenic plants. AM_1A-1-5 and AM_2-2-2 leaves showed the highest increments in BCAAs (Figure S3). For AM_1A-1-5, increments were 2.7-, 4.7- and 6.7-fold WT for Leu, Ile and Val, respectively. The levels of glucose and fructose as well as organic acids (malate, citrate and glutamate) were also analysed. We did not detect significant differences between the wild-type and cisgenic tomatoes in terms

Table 1 Absolute quantification of BCAAs in flesh of three independent AM and three independent WT fruits collected at 7 dpb

	WT-1	WT-2	WT-3	AM-1	AM-2	AM-3
Isoleucine (μg g^{-1} FW)	16.23 ± 1.43	16.26 ± 1.73	10.63 ± 0.30	42.76 ± 2.70	49.38 ± 1.75	43.54 ± 1.72
Leucine (μg g^{-1} FW)	17.73 ± 1.70	17.48 ± 1.73	11.29 ± 0.20	299.78 ± 17.68	358.26 ± 18.60	306.58 ± 10.22
Valine (μg g^{-1} FW)	14.82 ± 0.91	14.61 ± 2.12	10.57 ± 0.47	99.85 ± 21.12	137.04 ± 2.11	120.80 ± 3.43

of organic acid levels. Interestingly, glucose and fructose were found in significant higher levels in the cisgenic tomatoes (Figure S4).

Branched chain volatiles (BCVs) share with BCAAs the same biosynthesis metabolic pathway (Kochevenko *et al*., 2012; Rambla *et al*., 2014). This group of volatiles is highly relevant for tomato fruit liking as, of the circa 30 volatiles considered to be involved in the human perception of tomato flavour, 8 of them are BCVs: 3-methylbutanenitrile, 1-nitro-3-methylbutane, 3-methylbutanoic acid, 3-methylbutanol, 3-methylbutanal, 2-isobutylthiazole, 2-methylbutanol and 2-methylpropyl acetate (Tieman *et al*., 2017). Twelve BCVs were identified in the fruit via GC–MS. Four of them, 3-methylbutanal, 3-methylbutanenitrile, 3-methylbutanoic acid and 1-nitro-3-methylbutane, were significantly increased in the three cisgenic lines. The biosynthesis of these four volatiles is related to the BCAA leucine, which was also the amino acid with the most altered content. The most dramatically altered BCVs were 1-nitro-3-methylbutane and 3-methylbutanoic acid, respectively, showing up to 91.5- and 26.3-fold increments as compared to the WT (Figure 2d). These increases are remarkably higher than those observed in the BCAAs, thus suggesting a looser control in the accumulation of these leucine-related volatiles as compared to their respective amino acid. Other BCVs, including all those related to isoleucine and valine, showed either significant increase only in some of the lines or nonsignificantly altered levels (Figure S5).

Fruit-specific expression of SlMYB12 enhances flavonoids production

The characterization of the cisgenic lines proceeded with the validation of the functionality of the second TU present in the AM lines (pE8::SlMYB12::tE8). We first verified the E8-mediated ripening-dependent expression of the cisgenic SlMYB12 gene (cMYB12), using RT-qPCR primers pairs that discriminate between cisgenic and endogenous (eMYB12) copies (see Figure S2). As expected, cMYB12 was almost undetectable in green fruits and its levels sharply increased at breaker (220-fold increment), reaching a maximum at orange stage and decreasing at later maturation stages (Figure 3a). In comparison, eMYB12 expression showed smaller variations with ripening (13-fold increase from green to breaker), following a decreasing trend at later stages (Figure 3a). While cMYB12 could be detected both in the flesh and in the peel of cisgenic fruit, eMYB12 was only expressed in the peel, both in WT and AM fruits (Figure S6).

As a final characterization step, we analysed the content of several compounds in the flavonoid pathway (Figure 3b) in AM and control fruits. For this, red ripe fruits from three different cisgenic plant lines (1B-4-2, 1A-1-5 and 2-2-2), together with fruits of their corresponding azygous lines (1B-4-5, 1A-1-3 and 2-2-7, respectively), and a control wild-type plant were separately analysed by LC-ESI (+/−)-MS, and the most relevant flavonoids were identified and quantified relative to the WT. Overall, significant changes affected either typical most abundant compounds (e.g. naringenin chalcone, rutin) than minor components (kaempferol, quercetin, etc.) of the tomato fruit (Slimestad *et al*., 2008; Stewart *et al*., 2018). More in detail, major differences were observed in several specific flavonol aglycones in line AM_1B-4-2, where quercetin, myricetin, morin and kaempferol showed over-accumulation levels of 40-, 10-, 400-, 2- and 70-fold, respectively (Figure 3c). Significant over-accumulations were also found for glycoside derivatives of these compounds, including rutin, kaempferol-diglucoside, kaempferol-dihexose, kaempferol-dihexose deoxyhexose, kaempferol-glucose rhamnose, kaempferol-glucosyl-glucoside-rhamnoside, kaempferol-rutinoside, quercetin hexose and quercetin deoxyhexose-hexose-deoxyhexose (Figure 3c and Figure S7). In addition to flavonols, other flavonoid compounds including chalcones such as naringenin chalcone hexose, naringenin chalcone dihexose, naringenin chalcone glucoside, hydroxylated naringenin chalcone, flavanones such as eriodictyol-hexose and methyl-ether of (eriodictyol/eriodictyol chalcone) hexose, and flavones such as luteolin were also over-accumulated in AM fruits (Figure 3d and Figure S7).

As Luo et al. reported modifications on caffeoyl-quinic acids (CQAs) levels when AtMYB12 was expressed in tomato fruits (Luo *et al*., 2008), we decided to investigate whether these compounds were also modified in the AM lines. We observed a modest 2.9- and 2.6-fold enrichment of caffeic acid for lines 1B-4-2 and 1A-1-3, respectively, while for line 2-2-2 no substantial modification on caffeic acid levels was detected. For all three lines, an increment in caffeic acid hexose IV of roughly twofold was observed. However, no differences were observed in any of the lines for the CQAs content. Finally, we carried out absolute quantification of most relevant flavonol aglycones quercetin and kaempferol using hydrolysed fruit extracts. As shown in Table 2, the enrichment of fruits in this type of compound was confirmed in absolute values quantifications, with remarkable accumulations that reach up to 100 μg g^{-1} FW of quercetin in ripe fruits.

Metabolic fingerprinting clearly separate AM tomatoes from WT both in peel and flesh

A general view on how this new metabolic engineering approach affected the overall fruit metabolite composition was obtained by means of an untargeted LC–MS metabolomic profiling. Principal component analysis (PCA) of metabolic features detected in flesh and peel samples collected at 4 and 7 dpb showed a clear discrimination between AM and WT samples (Figure 4a). Class separation becomes more evident at the later ripening stages both in peel and flesh, as expected for the ripening-associated expression programmed for Myb12. A heatmap of the 100 most statistically different features showed that the most relevant changes observed in fruits corresponded to metabolites whose relative abundance increases in AM fruit, while only a few features increased their relative accumulation levels in the WT (Figure 4b). This bias towards metabolite accumulation occurs in

flesh samples both at 4 dpb and at 7 dpb. In contrast, the direction of changes seemed more balanced in peel, with even a majority of down-regulated features observed at 7 dpb. A Venn diagram of features that were differentially overexpressed in AM shows that approximately 80% of the differential features were present in the flesh and a 68% of them in the peel (Figure S8). Among the features overexpressed in WT, the 83% of them are present in the peel and the 66% in the flesh. 136 (30%) were

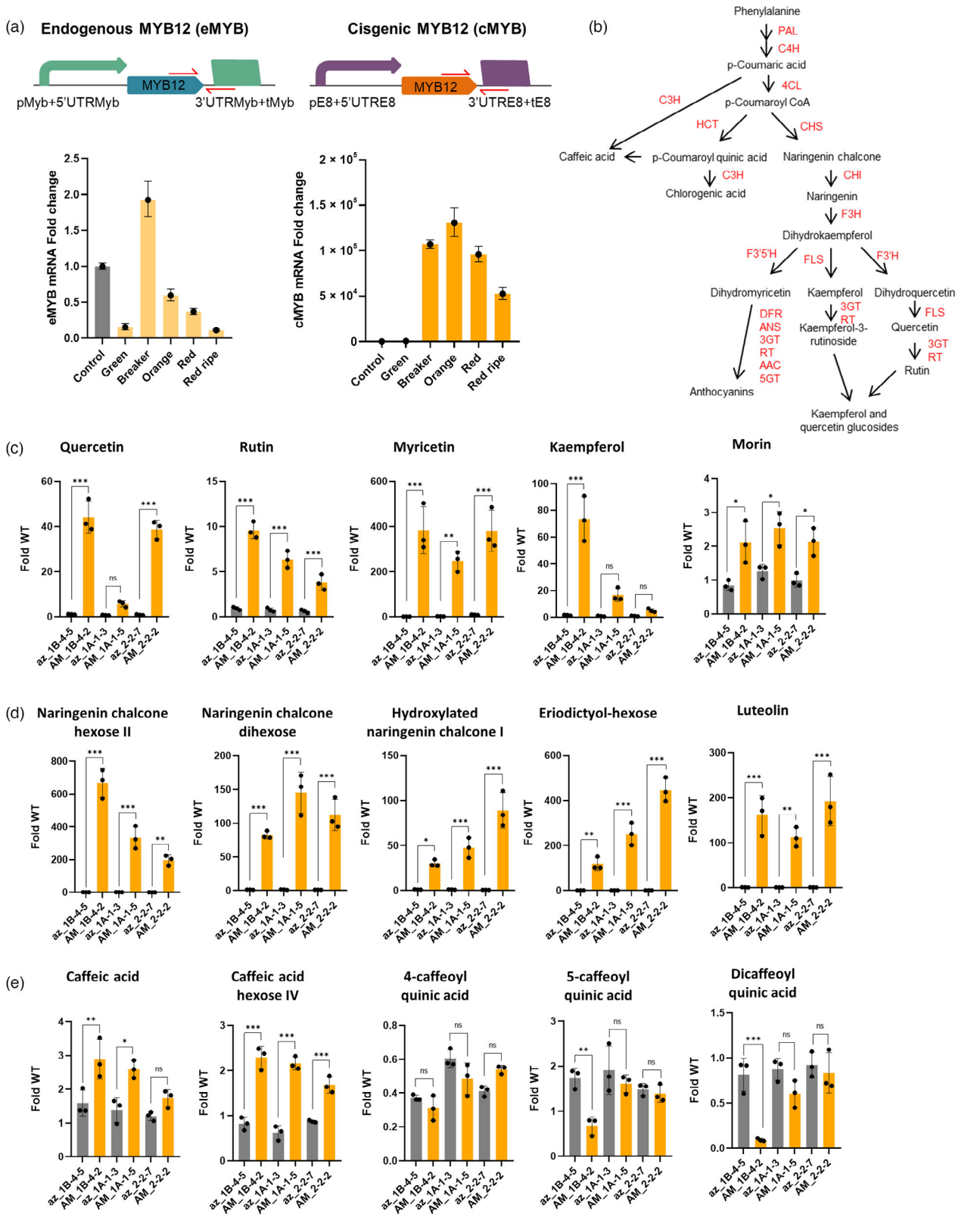


Figure 3 Fruit-specific expression of SlMYB12 results in increased phenylpropanoids content. (a) Primer design for differential detection of the endogenous and the cisgenic Solanum lycopersicum ALS mRNA (top) and mRNA fold change of endogenous and cisgenic ALS (bottom). Fruits of a T3 2-2-2 homozygous plant were harvested at different ripening stages. The control sample is a fruit of an azygous line harvested at breaker. Error bars represent standard deviation of technical replicates ($n = 3$). (b) Schematic representation of the phenylpropanoids biosynthetic pathway. (c) Fold change on the flavonols content of red ripe tomato fruits of three independent T2 AM lines compared to their corresponding azygous lines determined using HPLC. (d) Fold change on other flavonoids content of red ripe tomato fruits of three independent T2 AM lines compared to their corresponding azygous lines determined using HPLC. (e) Fold change on the caffeoyl quinic acids content of red ripe tomato fruits of three independent T2 AM lines compared to their corresponding azygous lines determined using HPLC. Asterisks indicate values that are significantly different *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) from their corresponding azygous lines (Student's *t*-test). Error bars represent SD of independent biological replicates ($n = 3$).

Table 2 Absolute quantification of flavonols in flesh of three independent AM and three independent WT fruits collected at 7 dpb

	WT-1	WT-2	WT-3	AM-1	AM-2	AM-3
Quercetin (μg g^{-1} FW)	1.73 ± 0.03	2.00 ± 0.07	1.84 ± 0.05	97.53 ± 3.16	80.96 ± 4.52	74.85 ± 2.27
Kaempferol (μg g^{-1} FW)	0.216 ± 0.004	0.259 ± 0.001	0.242 ± 0.019	14.43 ± 0.67	16.65 ± 0.60	14.70 ± 1.55

present in flesh and peel collected at 7 dpb and 108 (24%) were present only in the peel at 7 dpb.

To obtain a better insight on the identity of those features showing the most differential behaviour, we performed a new analysis employing more resolutive analytical conditions facilitating compounds identification. We chose 7 dpb samples for this new detailed analysis as this time point showed the most dramatic differences. The results of this new analysis are shown in Figure 4c,d. Consistently with previous untargeted fingerprint, PCA analysis of 7 dpb samples showed clear separation among samples categories. The first component (51.4%) separated flesh from peel samples, whereas the second component (36.6%) clearly separated samples by genotype. The hierarchical clustering of 60 most differential metabolites shown in Figure 4d was also consistent with the differences observed in targeted analysis (see Table S2). This hierarchical clustering identified a first cluster of compounds with a strong overaccumulation in both peel and flesh of cisgenic fruits. This cluster is formed by flavonoid compounds (naringenin and kaempferol glycosides) and most interestingly, several features identified as acyl glycosides whose acyl chain derives from Leucine (Table S2). A second cluster that shows strong overaccumulation in cisgenic fruit flesh contains hydroxycinnamic acid glycosides and, remarkably, leucine. A third large cluster of compounds accumulating preferentially in both AM tissues was identified in the heatmap, representing a wider variety of compounds in which flavonoids and hydroxycinnamic acid derivatives were predominant. Finally, only a few compounds, among those most differentially regulated, were found to accumulate preferentially in WT tissues. These correspond to glycoalkaloids in WT peel and hydroxybenzoic acid derivatives in both WT tissues (Figure 4d).

Transcriptomic analysis of AM fruits revealed a general activation of flavonoids and branched amino-acid pathways

Further investigation of the effects of simultaneous expression of ALS and MYB at high levels in the fruit was carried out using transcriptomic analysis. Tomato fruits of AM and WT plant lines were harvested at 4 dpb and flesh samples were used for RNAseq analysis. In the flesh, a total of 1874 genes were differentially expressed, a majority of then (1200) overexpressed in the AM fruits (Table S3). Interestingly, SlALS ranked second among the most significant up-regulated genes. We estimated that in the cisgenic line, ~99.2% of reads corresponded to the cisgenic copy and only ~0.8% of them to the endogenous one. GO term enrichment analysis revealed that genes involved in the small molecule, carboxylic acid, oxoacid, organic acid, nucleoside/nucleotide and cellular amino acid biosynthesis processes were significantly overrepresented in the AM fruits (Figure 5a and Table S4). Regarding the cellular component, the GO terms significantly overrepresented in the AM lines were mainly related to the plastids and their different compartments (Figure 5a and Table S4). The list of genes contributing to these GO terms include the acetolactate synthase and several plastidial kinases and oxidoreductases involved in the shikimate and phenylpropanoids pathways (see Table S5).

A KEGG enrichment analysis confirmed the biosynthesis of secondary metabolites as the most significantly enriched biological process, next to the amino acids biosynthesis. A closer look to the flavonoid pathway revealed a total of 14 up-regulated genes in AM fruits, distributed in multiple steps of the pathway (see Figure 5b). Among them, the strongest up-regulation occurred in chalcone synthases 1 and 2 (CHS1 and CHS2), the naringenin 3-dioxygenase (F3H), the chalcone isomerase 1 (CHI1) and the flavonol synthase (FLS) (Figure 5c). This latter gene, catalysing the oxidation of dihydroflavonols to produce flavonols, showed the most dramatic up-regulation, in accordance with the observed increase of kaempferol, quercetin and myricetin. The KEGG analysis also revealed that 50 genes involved in the amino acid biosynthesis pathway were differentially expressed in the AM fruits, most of them up-regulated (Figure S9). Interestingly, those up-regulated genes in the shikimate pathway leading to the biosynthesis of phenylalanine were also identified by Zhang *et al.* (2015) upon ectopic expression of AtMyb12 driven by E8 promoter, whereas others leading to BCAAs synthesis are exclusive of the combination of SlMyb12 and SlALS.

Discussion

Cisgenesis refers to those genetic engineering approaches that make use only of DNA elements derived from the genetic pool of

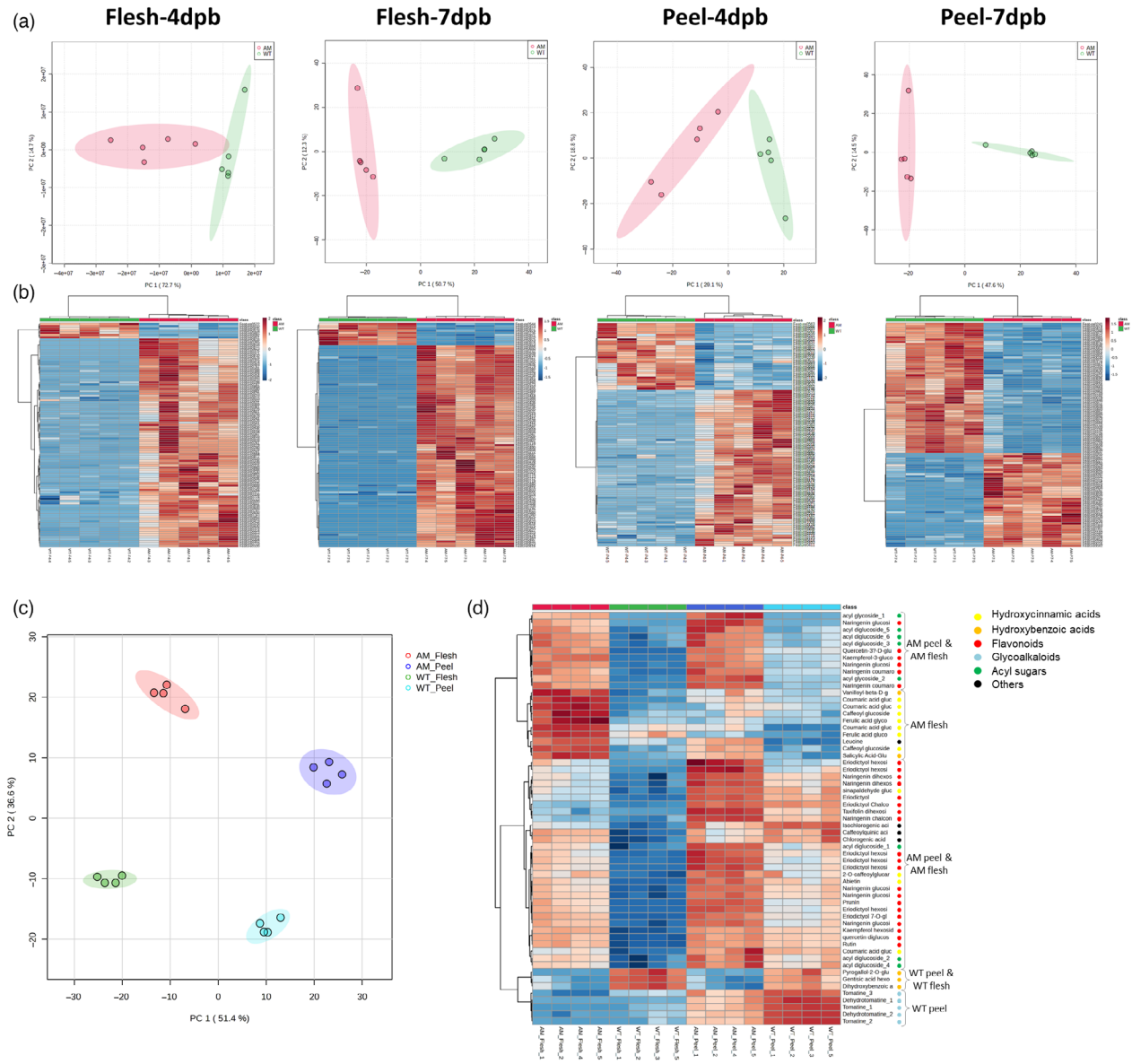


Figure 4 Metabolite profiling with LC–MS of ALS-MYB flesh and peel samples. (a) Principal component analysis (PCA) of AM versus WT samples. (b) Heatmap of the 100 most differential features detected in AM and WT flesh and peel samples. (c) Principal component analysis (PCA) of AM versus WT samples collected at 7 dpb and analysed with LC–MS. (d) Heatmap with the most differential compounds upon curation detected in AM and WT flesh and peel samples at 7 dpb.

sexual compatible species. Cisgenic crops are intrinsically genetically modified organisms (GMOs), and therefore, they are sensibly regulated as such (van Hove and Gillund, 2017). However, bearing in mind the reduction in perceived risk associated with the avoidance of exogenous DNA sequences in the makeup of cisgenic plants by consumers, it is reasonable to claim and to expect a reduction in the regulatory constraints governing their consumption and/or commercialization, especially in those countries where process-based GMO regulations prevail (Russell and Sparrow, 2008). Interestingly, several surveys show that public acceptance is higher for crops generated using new breeding technologies that avoid the presence of foreign DNA sequences (Delwaide *et al.*, 2015). Recently, the EU Commission conducted a public have-your-say enquiry on the need to change regulations for plants produced using new breeding techniques, a survey that was focused on genome editing but that extended to cisgenesis. The expectation for regulatory changes has brought along a renewed interest in the evaluation of cisgenesis-based engineered crops. An immediate application of cisgenesis is the enrichment of food composition in health-related compounds, more specifically in those compounds that are already present in the crop, but for which genetic intervention could result in a higher abundance or a more convenient relative concentration. The cisgenic substitution of the regulatory sequences governing the expression of transcription factors (TFs) or biosynthetic enzymes (BEs) are two strategies to modify metabolic fluxes, leading to the accumulation of health-promoting compounds. Here, we combined the TF and BE approaches in a single cisgenic intervention in tomato fruits, demonstrating the feasibility of combined fortification strategies.

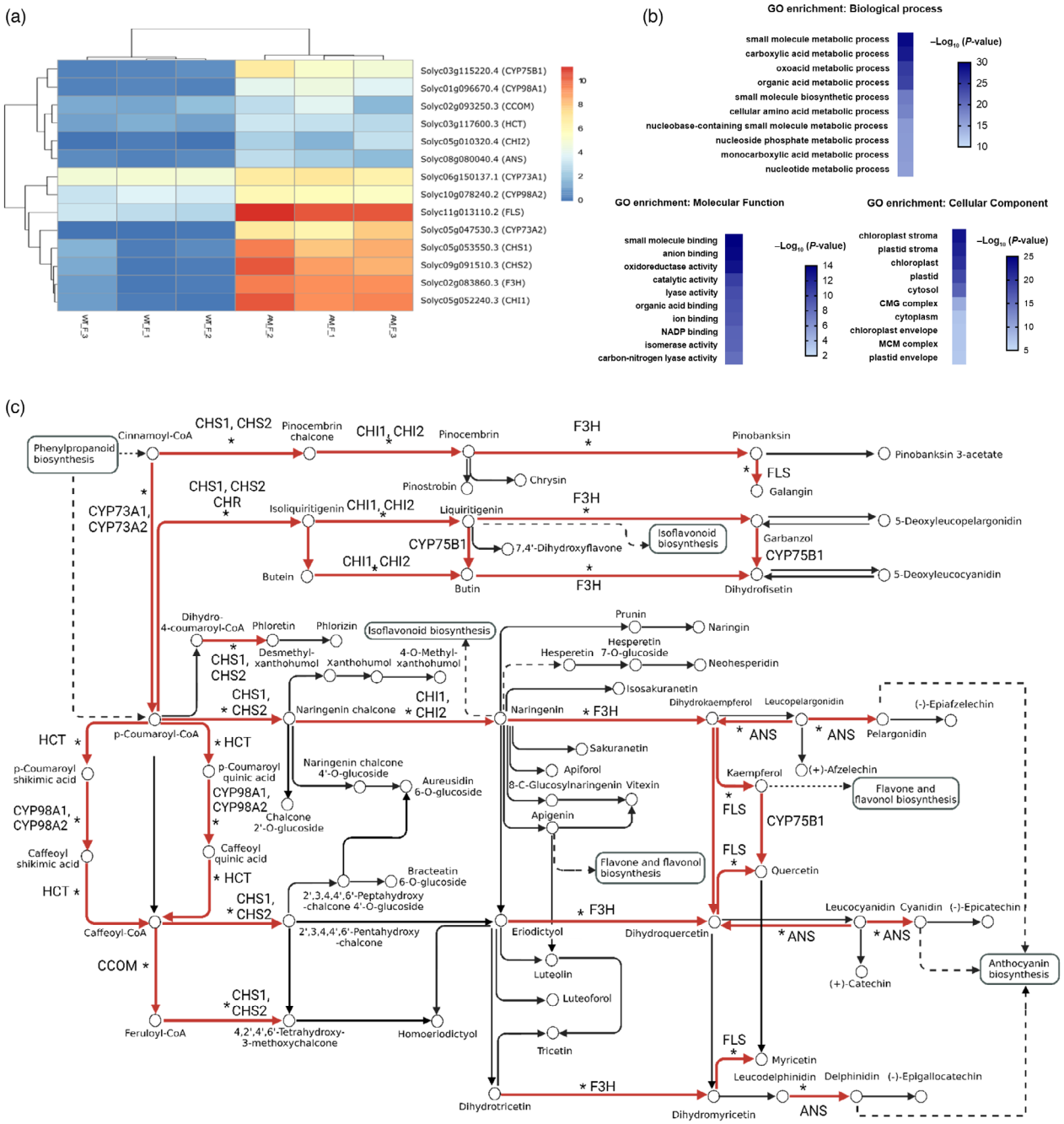


Figure 5 Transcriptomic analysis of ALS-MYB flesh samples shows overexpression of multiple genes involved in the amino acids and flavonoids biosynthetic pathways. (a) GO terms enrichment analysis of genes overexpressed in the AM fruits. The ten most significant GO terms are displayed. (b) A KEGG analysis shows that several genes of the flavonoids pathway (red arrows) are overexpressed in the AM fruits compared to WT. Asterisks indicate genes overexpressed in AtMYB12 fruits from Zhang *et al.* (2015). (c) Log FPKMs of the genes differentially expressed among lines in the flavonoids pathway depicted in (b).

The herbicide-resistance trait conferred by mutations in ALS loci has been used traditionally in many crops (Okuzaki *et al.*, 2007; Shimizu *et al.*, 2008), with resistant mutations known to appear spontaneously in weed species (Tranel and Wright, 2002). The employ of ALS as transgenic selection marker is also common (Li *et al.*, 1992; Yao *et al.*, 2013), but its use in cisgenic breeding requires species-specific adaptation, limiting its widespread use for this particular purpose. Here, we showed that the 186 Pro > Ser conversion (197 in reference to AtALS) of SlALS confers resistance to chlorsulfuron, as previously demonstrated for the Arabidopsis ALS in tobacco (Haughn *et al.*, 1988) and with the apple ALS both in tobacco and apple (Yao *et al.*, 2013). The transformation efficiencies of 5.5% reported here were lower than those obtained by the use of the well-established nptII antibiotic selection marker but were high enough to be considered for routine transformations, particularly if the cisgenic status conferred a commercial advantage to the resulting product. To our knowledge, no other ALS-based purely cisgenic

breeding strategy has been described so far. Surprisingly, an associated increase in BCAAs has not been reported before, despite the extended use of this marker in transgenic selection strategies. A possible explanation is that the levels of expression of the mutated ALS in target food tissues were considerably lower to those conferred by the Mtb promoter employed here. In other examples such as in apple (Yao *et al*., 2013), where ALS expression was driven by a CaMV35S promoter, the effect on BCAAs might have remained unnoticed. When the goal is to obtain the double biofortification, the choice of a strong promoter seems the best way to proceed; however, in the light of our findings, the employment of ALS under a strong promoter should be reconsidered when the aim is to generate new plants varieties free of effects related to the use of the selectable marker. For these cases, the use of endogenous ALS promoter could be an advisable strategy. Conversely, ALS-based BCAA enrichment could be also considered as an interesting biofortification strategy to apply to protein-rich crops to modify the profile of amino-acids in plant-based foods. Our results also suggest that transaminases and other enzymes involved in the BCV synthesis are not limiting factors, and by simply increasing the pool of BCAA precursors, higher levels of BCVs can be obtained. Furthermore, we also observed strong accumulation in BCAAs- derived volatiles in leaves of cisgenic tomato lines. Since E8-driven gene expression is strictly fruit-specific, these observations strongly suggest that the observed BCAAs accumulation is to a large extent, if not exclusively, a consequence of ALS overexpression. This point is reinforced by data generated by Breitel *et al*. (2020), which showed that the levels of Ile, Leu, and Val in the AtMYB12 fruits increased on average 1.3-, 1.8- and 1.4-folds, respectively as compared to the wild-type fruit, with none of these changes found to be statistically significant. In comparison, these are clearly minor variations when contrasted to the 3.8-, 14.0- and 7.8-fold changes found, respectively, in dually fortified fruits, with all increases being statistically significant.

As expected, the parallel overexpression of SlMYB12 driven by E8 promoter resulted in a notable over-accumulation of specific compounds of the flavonol biosynthesis pathway in the fruit, in line with previous results reported with transgenic lines overexpressing the orthologous transcription factor from Arabidopsis (Luo *et al*., 2008). All three independent lines analysed here showed a drastic increase of naringenin, kaempferol and quercetin glycosides in the fruit in reference to wild type and the corresponding azygous lines. The degree of enrichment in target metabolites obtained with cisgenesis seems comparable than that reported in equivalent transgenesis, despite the use of endogenous TF and the concomitant enrichment in BCAAs. Overaccumulations of most relevant aglycon flavonols such as quercetin and kaempferol measured from hydrolysed fruit extracts to enable absolute quantification are close to 50-fold as compared with wild-type Moneymaker fruits, a similar range to what was found earlier in transgenic tomatoes transformed with E8-driven Arabidopsis AtMYB12 gene (Zhang *et al*., 2015), who established earlier the role of MYB12. Also in this previous study, Zhang *et al*. (2015) showed that AtMYB12 under the control of the E8 promoter resulted in transcriptomic changes in nearly all the genes involved in glycolysis, the pentose phosphate, the shikimate and the flavonoid pathways. Our data indicate that SlMYB12 is functionally equivalent to AtMYB12. We observed that, like AtMYB12, SlMYB12 has a role in the transcriptional activation not only of phenylpropanoid biosynthetic enzymes but also in rewiring carbon flux towards the production of aromatic amino acids. As expected, a correlation between up-regulated transcripts and metabolite levels was found for most of the flavonoid pathway steps when data were analysed in the context of metabolic pathways.

In addition to the expected enrichment in targeted compounds, untargeted metabolomic profiles showed a clear separation between cisgenic and control fruits. Separation among samples is more evident at the later ripening stage (7 dpb), arguably due to the late expression of the two cisgenes driven by the E8 and Mtb promoters, respectively. The identification of the 60 most significantly differential features yielded a fruit metabolic landscape fully congruent with what was observed in targeted analysis as a consequence of Myb12 overexpression, with flavonoids and hydroxycinnamic acids derivatives popping up among the most significantly up-regulated compounds. Interestingly, leucine and remarkably a group of compounds tentatively identified as acyl glycosides are among the most up-regulated compounds. These acyl glycosides are reported to derive from the branched chain ketoacids (Maeda, 2019), and their enrichment in ALS overexpressing tomatoes is, similarly to the accumulation of BCVs observed in GC–MS profiles (Figure 2d), a consequence of the increased BCAA levels in those lines and support the idea that BCAA are limiting factors in the production of both type of specialized metabolites.

The untargeted metabolome profiles shown here highlight the extent of the compositional modifications that can be obtained by simply introducing changes in the expression levels of two unique genes, and this is expected to happen regardless of the use of transgenesis, cisgenesis or traditional breeding. In our view, this reflects the level of interconnection that occurs among biosynthetic pathways and questions the applicability of the substantial equivalence concept (Catchpole *et al*., 2005) for the evaluation of safety profiles in plant breeding when nutritional enrichment is the objective. It would be more informative to compare the metabolomic profiles of the engineered crop with other varieties of the same or related crops (e.g. tomatoes in this case), an approach that could help to identify any suspicious, unintended deviation from what is generally considered as safe in the metabolic composition of the same range of product. In this regard, the availability of increasingly complete pan-metabolomes from different crop species is a highly valuable resource (Drapal *et al*., 2022; Enfissi *et al*., 2021). In our view, strong unintended deviations are especially unlikely to occur using cisgenic breeding approaches.

Although dually fortified in flavonols and BCAAs, the metabolite enrichment levels in terms of overall dietary requirements are not comparable for the two types of metabolites. The absolute levels of quercetin produced in cisgenic fruits reached remarkable levels of 100 μg g^{-1} FW, equivalent to 10 mg of quercetin in an average 100 g serving portion, a quantity close to that employed when quercetin is used as a food supplement. On the other hand, the content of BCAAs in a serving portion of tomato represents no more than 2%–5% of the daily dietary requirements (85 mg kg^{-1} according to Kurpad *et al*., 2006). In a strict vegetarian diet, antioxidant supplementation would therefore be likely covered by the normal daily intake of cisgenic tomatoes, but the levels of BCAAs would be insufficient in this single product. To be effective, a similar approach could be implemented in parallel in other fruit and vegetables, particularly if increases in free amino acids were also reflected in increases in amino acid composition in proteins in the vegetarian diet.

Multiple biofortification of crops has been successfully achieved earlier using transgenic approaches (Díaz-Gómez *et al*., 2017; Zhu *et al*., 2013). The tomato fruits described in this work are an example of how new breeding techniques can also be applied to customize food composition and adapt it to new needs that arise in modern societies, especially in the context of a steady reduction of the intake of meat-derived products for sustainability and/or ethical reasons. Certainly, any genetic modification influencing a metabolic pathway, whether transgenic, randomly induced via mutagenesis, sexually introgressed from compatible species or rationally designed using sequences from the breeder's pool, would induce certain level of impacts on other pathways. As shown here, our cisgenic approach induces detectable transcriptional changes in genes other than the introduced cisgenic genes and their direct targets, and most importantly, our nontarget metabolite analysis detects significant changes in the accumulation of 'untargeted' compounds besides BCAAs and flavonols. However, we showed that, at least in this intervention, the most relevant "unintended" changes are related to catabolic pathways acting downstream the targeted compounds (namely BCAA-derived volatiles and acyl glycosides derived from Leucine), and these changes could have been anticipated given our current knowledge of fruit metabolism. We conclude that the safety guaranties of a breeding intervention aiming at improving food nutritional composition are better served by providing exhaustive description of the metabolic composition in the context of a deep knowledge of the plant metabolism, rather than blindly relying on the type of technique employed in such intervention. The increasing availability of crop genomic and metabolic data will not only help us to understand crop genetics but also will provide us with new, well-defined DNA components that will enable cisgenic engineering of crop metabolism with increasing accuracy and safety.

Experimental procedures

Cloning procedures

All plasmids were assembled with GoldenBraid standard procedures (Sarrion-Perdigones *et al*., 2013). SlALS gene (Solyc03g044330.1) was amplified from MoneyMaker genomic DNA. Three nucleotide changes were introduced on the ALS coding sequence, two of them for removing internal BsaI and BsmBI restriction sites due to cloning requirements and a third mutation that leads the Pro > Ser amino acid change at position 186 conferring CLS resistance. To introduce the mutations on the SlALS and to create level 0 parts GB0816, GB0914 and GB0144, we followed the GB domestication standard procedures (Sarrion-Perdigones *et al*., 2013) with primers listed on Table S6. GB0080, GB0142 (Sarrion-Perdigones *et al*., 2013) and GB0075 (Vazquez-Vilar *et al*., 2015) had been adapted to the GB standard in previous works. Level 0 parts making the selection marker transcriptional unit (GB0080, GB0816 and GB0142) were assembled in the pDGB1α1R GBvector with a restriction-ligation reaction to create the level 1 transcriptional unit GB0818. In the same way, the MYB12 fruit-specific expression-cassette was assembled in the pDGB1α2 GBvector from Level 0 parts GB0075, GB0914 and GB0144. Finally, the selection marker and the MYB12 fruit-specific expression-cassette were combined in a binary reaction in the pDGB3Ω1 vector generating level >1 element GB0830.

Tomato transformation

GB0830 was transferred to *Agrobacterium tumefaciens* LBA4404 strain for stable tomato transformation. Tomato (var. MoneyMaker) transformation was carried out as described by Ellul *et al*. (2003) with minor modifications. Briefly, cotyledons of 10 days tomato plants were cut and explants were submerged in the Agrobacterium culture for half an hour. Next, they were transferred to coculture medium and kept in the dark for 48 h. Then, explants were transferred to the organogenesis medium with different doses of chlorsulfuron. Chlorsulfuron (purchased from Chemservice, N-11461-100 mg) was resuspended in a small volume of 1 M KOH and diluted to the desired final concentration with distilled water. Individual shoots were excised and transferred to elongation medium prior to be transferred to rooting medium for root regeneration.

Plant material

Wild-type and T2 and T3 AM plants were grown under natural light and controlled temperature conditions (24 °C during the day, 18 °C at night) in a greenhouse. As controls, WT plants and azygous (az) plants were employed for the different lines. Azygous controls are descendants from T1 heterozygous plants lacking T-DNA insertions. Three T2 AM lines (AM_1B-4-2, AM_1A-1-5 and AM_2-2-2) and their corresponding azygous lines (az_1B-4-5, az_1A-1-3 and az_2-2-7) were tested. For initial characterization including phenylpropanoids, volatiles and primary metabolites relative quantification, red ripe fruits were collected, frozen in liquid nitrogen, ground in a cryogenic mill and stored at −80 °C until analysis. In all other experiments T3 and T4 plants descendants of AM_2-2-2 were used. For RT-PCR, RNA-seq, untargeted metabolomics and absolute flavonol and BCAAs quantification, fruits were checked daily after reaching the mature green stage and collected at different days post-breaker depending on the analysis. For all the analyses except RT-PCR, the peel of three to five fruits was separated and pericarp and peel were independently frozen in liquid nitrogen, ground and stored at −80 °C until analysis.

Determination of ALS activity in leaves

The determination of the ALS activity was performed as described in Shimizu *et al*. (2008), with minor modifications. Tomato leaf discs (d = 1.5 cm) of 2-month-old plants were excised and incubated on plates with 25% MS medium containing 0.5 mM 1,1-cyclopropanedicarboxylic acid and with or without 50 μg L^{-1} chlorsulfuron. After 42 h, samples were frozen in liquid nitrogen and subsequently homogenized and extracted with 200 μL of 0.025% Triton X-100 followed by 15 min of centrifugation (12 000 × ***g***). Next steps were performed as indicated in Shimizu *et al*. (2008).

Determination of primary metabolites

The relative levels of polar metabolites were determined as described in Zanor *et al*. (2009)). One hundred mg of frozen tomato fruit powder were extracted in 1.4 mL of methanol and 60 μL of an aqueous solution with 0.2 mg mL^{-1} of ribitol, which was used as internal standard. Extraction was performed at 70 °C for 15 min in a water bath. The extract was centrifuged at 14 000 rpm for 10 min, and the supernatant was recovered and fractionated adding chloroform and Milli-Q water. After vigorous vortexing and centrifugation at 4000 rpm for 15 min, 50 μL of the aqueous phase was recovered and dried overnight in a speed-vac. The dry residue was subjected to a double derivatization procedure with methoxyamine hydrochloride (20 mg mL^{-1} in pyridine, Sigma) and *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (Macherey-Nagel). Fatty acid methyl esters (C_8–C_{24}) were

added and used as retention index (RI) markers. Analyses were performed on a 6890 N gas chromatograph (Agilent Technologies) coupled to a Pegasus 4D TOF mass spectrometer (LECO). Chromatography was performed with a BPX35 (30 m, 0.32 mm, 0.25 μm) capillary column (SGE Analytical Science) with 2 mL min^{-1} constant helium flow. Oven programming conditions were as follows: 2 min of isothermal heating at 85 °C, followed by a 15 °C min^{-1} temperature ramp up to 360 °C. Injection temperature was set at 230 °C, and the ion source was adjusted to 250 °C. Data were acquired after electron impact ionization at 70 eV and recorded in the 70–600 m/z range at 20 scans s^{-1}. Chromatograms were analysed by means of the ChromaTOF software. Metabolites were identified by comparison of both mass spectra and retention time with those of pure standards injected under the same conditions (Table S1). Peak area of each identified compound was normalized to the internal standard area (ribitol) and sample dry weight. Five replicates per line were performed.

Absolute quantification of valine, leucine and isoleucine

The absolute quantification of the branched chain amino acids was performed as described in Methods S1.

Determination of volatile compounds

Volatile compounds were analysed as described in Methods S2.

LC-ESI(+/−)-MS analysis of tomato fruit phenylpropanoids

Phenylpropanoid extraction was carried out as previously described (Fasano *et al.*, 2016). Briefly, 10 mg of ground freeze-dried fruit powder was extracted with 0.75 mL cold 75% (v/v) methanol, 0.1% (v/v) formic acid, spiked with 10 μg mL^{-1} formononetin. Samples were vortexed for 30 s, shaken for 15 min at 15 Hz using a Mixer Mill 300 (Qiagen) and kept at RT for 5 min (twice). After centrifugation for 15 min at 20 000 × ***g*** at 4 °C, 0.6 mL of supernatant was removed and transferred to HPLC tubes. For each genotype, extractions from at least 4 fruits were performed. Separation was carried out using an Ultimate 3000 HPLC coupled to a Q-EXACTIVE mass spectrometer (ThermoFisher) equipped with a C18 Luna reverse-phase column (150 × 2.0 mm, 3 μm; Phenomenex, Macclesfield, UK) and a gradient system as follows: 95%A:5%B for 1 min, followed by a linear gradient to 25%A:75%B over 40 min. LC conditions were kept for 2 more minutes, before going back to the initial LC conditions in 18 min. Ten μL of each sample was injected and a flow of 0.2 mL was carried out during the whole LC runs. Detection was performed continuously from 230 to 800 nm with an online Ultimate 3000 photodiode array detector (PDA, Thermo Fischer Scientific, Waltham, MA). All solvents used were LC–MS grade quality (CHROMASOLV® from Sigma-Aldrich). The Exactive Plus Orbitrap mass spectrometer was equipped with a heated electrospray probe (H-ESI). ESI and MS parameters were as follows: spray voltage −5.0 kV, sheath gas and auxiliary nitrogen pressures 30 and 10 arbitrary units, respectively; capillary and heater temperatures were set at, respectively, 250 and 150 °C, while tube lens voltage was 50 V. Data were acquired in profile mode. Identification was performed as reported before (Diretto *et al.*, 2020), through (i) comparison of chromatographic and spectral properties of authentic standards and reference spectra, if available; (ii) on the basis of the full MS data, using m/z accurate masses, as reported on Pubchem database (http://pubchem.ncbi.nlm.nih.gov/) for monoisotopic masses identification, or on Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/) in case of adduct ion detection; and/or (iii) by comparing theoretical and experimental MS fragmentation (by MS/MS) as reported in public (e.g. Metlin) or in house databases. Metabolites were relatively quantified on the basis of the internal standard amounts and data were subsequently normalized, for each metabolite, on WT levels. Detailed information on metabolite data detection, including chemical formula, RT, ionization characteristics and their level of identification, is now presented as Table S1. ANOVA was applied to the absolute metabolite quantifications, and pair-wise comparisons were used in Tukey's HSD test running on the Past 3.x software (http://folk.uio.no/ohammer/past/).

Flavonol absolute quantification

The absolute quantification of flavonols quercetin and kaempferol was performed as described in Methods S3.

LC–MS untargeted analysis for metabolic fingerprinting

Flesh and peel samples of five AM and five WT fruits collected at 4 and 7 dpb were frozen in liquid nitrogen and ground into a fine powder. Next, samples were freeze-dried and weighed (10 ± 0.5 mg) and extracted in methanol/water (1:1) as described previously. To each aliquot of the polar phase (100 μL), internal standard (genistein, 1 μg) was added. The polar phase was used for metabolite profiling by UPLC-ESI-QToF 6560 (Agilent Technologies, Stockport, UK) as previously described (Drapal *et al.*, 2022). Data processing was performed with Agilent Profinder (v10.0 SP1; Agilent Technologies, Inc.). The identified molecular features were relatively quantified to the internal standard and dry weight of the sample. Only molecular features that were present in at least two out of the five biological replicates for either AM or WT were analysed. PCA and hierarchical clustering analysis were performed with Metaboanalyst (Pang *et al.*, 2022) using auto scaling for data normalization.

LC–MS untargeted analysis for compound identification

Flesh and peel samples of four AM and four WT fruits collected at 7 dpb were used for this analysis. Sample extraction and chromatography were performed as described for flavonol quantification in Methods S3. Ionization was performed with heated electrospray ionization (H-ESI) in positive and negative mode. Samples were acquired in full scan mode (resolution 120 000) and mixes in both full scan and data dependent acquisition (DDA) to help with compound identification. For DDA, resolution was set at 30 000 and intensity threshold at 2e5. m/z range was from 150 to 1500. Peak identification and annotation and data analysis and statistics were performed with Compound Discoverer 3.3 software (Thermo Scientific, Waltham, MA) and Metaboanalyst (Pang *et al.*, 2022). For a first clean-up of the raw data, only features with peak rating >5 for at least 4 samples, assigned formula containing only C, H, N and O and at least one match in database searches were kept and subjected to statistical analysis. After ANOVA and *t*-test analyses, 658 features with log_2FC >1 and adj *P*-value <0.05 (Benjamini-Hochberg correction) for at least one comparison were kept. After manual curation of the data (peak selection based in retention time, MS/MS data, mzCloud and internal database matches and literature search), a final inclusion list of 59 metabolites was obtained (Table S2).

RNA isolation, RT-qPCR and RNA sequencing

For RT-qPCR analyses, fruits from AM plants were harvested at different ripening stages and frozen and ground using liquid nitrogen. Total RNA was extracted with TRIzolTM Reagent following manufacturer instructions. Four μg of total RNA was treated with a DNase-I Invitrogen Kit following manufacturer's instructions. Then, 1 μg of treated RNA was used for cDNA synthesis using a PrimeScript™ RT-PCR Kit (Takara, Kusatsu, Japan) in a final volume of 20 μL. Als and Myb12 expression levels were measured in three technical replicates reactions in the presence of a fluorescent dye (SYBR® Premix Ex Taq) using QuantStudio 3 equipment (Applied Biosystems, Waltham, MA). The specific primers for detection of endogenous and cisgenic AlS and Myb12 genes are listed in Table S1. Actin was used as an internal reference gene. Calculations were carried out according to the comparative ΔΔCt method. For transcriptomic analysis, both WT and AM fruits were marked at breaker and fruits were collected 4 days after breaker. Fruit flesh was frozen and ground using liquid nitrogen. Total RNA was extracted with TRIzolTM Reagent following manufacturer instructions. Both library preparation and RNA-seq sequencing were performed by Novogene (China) with total RNA, generating Paired end (PE) 2 × 150 bp reads with NovaSeq 6000 PE150 platform.

RNA-seq preprocessing and expression profiles analysis

Sequence reads were quality checked using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were quality trimmed and Illumina adaptors were removed with Trimmomatic v0.39 (Bolger *et al*., 2014). Clean reads were mapped against *Solanum lycopersicum* reference genome SL4.0 (Hosmani *et al*., 2019) using Hisat2 v2-2.2.1 (Kim *et al*., 2019), and gene abundances were calculated using StringTie v2.1.6 (Pertea *et al*., 2015) with the genome annotation ITAG4.0 (Hosmani *et al*., 2019). From these counts, a gene expression table of raw read counts was generated. Raw gene expression was normalized by trimmed mean of M-values and differential expression analysis was performed using edgeR v3.36.0 with a negative binomial exact test using both common and tagwise dispersion (Robinson *et al*., 2010). Then, significant differential expressed genes were kept if their false discovery rate (FDR) was <0.05 and their average expression for all samples was equal or greater than 1 count per million mapped (CPM). Kept genes were mapped against KEGG terms database using blastkoala (Kanehisa *et al*., 2016). The number of reads corresponding to endogenous and cisgenic MYB12 and ALS was calculated using single-nucleotide differences by allele counting using samtools mpileup v1.7 (Li, 2011).

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Conflict of interest

The authors declare that they have no conflict of interest.

Data availability statement

RNA-Seq data reported in this article are available at NCBI database (Bioproject PRJNA821387). Raw LC–MS data have been deposited in MetaboLights (study: MTBLS7657).

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Colorimetric assay for the determination of ALS activity in leaf extracts of three independent T2 AM lines and their corresponding azygous lines in the absence and presence of 50 μg L^{-1} chlorsulfuron. Red indicates ALS activity.
Figure S2 PCRs for specific amplification of the endogenous and transgenic copies of the ALS and MYB12 genes.
Figure S3 Overexpression of the acetolactate synthase gene results in increased branched chain amino acids in leaves.
Figure S4 Fold change on primary metabolites content of red ripe tomato fruits of three independent T2 AM lines compared to their corresponding azygous lines determined using HPLC.
Figure S5 Overexpression of the acetolactate synthase gene results in unintended increased branched chain volatiles in tomato fruits.
Figure S6 Expression levels of endogenous and cisgenic ALS and MYB12 genes in tomato fruit in different tissues.
Figure S7 Fruit-specific overexpression of the SlMyb12 gene results in increased phenylpropanoids in tomato fruits.
Figure S8 Venn diagram showing the number of independent and common features among the different samples identified with a metabolite profiling using LC–MS.
Figure S9 Transcriptomic analysis of ALS-MYB flesh samples shows overexpression of multiple genes involved in the amino acids biosynthetic pathway.
Table S1 Targeted metabolomics analysis of phenylpropanoid compounds in WT and ALS-MYB tomato fruits at red ripe (RR) stage.
Table S2 Untargeted metabolomics analysis in WT and ALS-MYB tomato fruits at 7 days post-breaker.
Table S3 List of differentially expressed genes between AM and WT flesh samples.
Table S4 List of GO terms with top significance retrieved from the RNAseq analysis of the AM fruits.
Table S5 Top 25 genes overexpressed in the AM fruits contributing to the chloroplast and plastid-related GO terms of the cellular component.
Table S6 List of primers used in this work.
Methods S1 Absolute quantification of valine, leucine and isoleucine.
Methods S2 Determination of volatile compounds.
Methods S3 Flavonol absolute quantification.