Manipulation of \( \beta \)-carotene levels in tomato fruits results in increased ABA content and extended shelf-life

Gianfranco Diretto  
*Casaccia Research Center*, gianfranco.diretto@enea.it

Sarah Frusciante  
*Casaccia Research Center*

Claudia Fabbri  
*Sapienza University of Rome*

Nicolas Schauer  
*Max-Planck-Institute*

Lucas Busta  
*University of Nebraska - Lincoln*, lbusta@unl.edu

See next page for additional authors

Follow this and additional works at: [https://digitalcommons.unl.edu/plantscifacpub](https://digitalcommons.unl.edu/plantscifacpub)

Part of the [Plant Biology Commons](https://digitalcommons.unl.edu/plantscifacpub), [Plant Breeding and Genetics Commons](https://digitalcommons.unl.edu/plantscifacpub), and the [Plant Pathology Commons](https://digitalcommons.unl.edu/plantscifacpub)

Diretto, Gianfranco; Frusciante, Sarah; Fabbri, Claudia; Schauer, Nicolas; Busta, Lucas; Wang, Zhonghua; Matas, Antonio J.; Fiore, Alessia; Rose, Jocelyn K. C.; Fernie, Alisdair R.; Jetter, Reinhard; Mattei, Benedetta; Giovannoni, Jim; and Giuliano, Giovanni, "Manipulation of \( \beta \)-carotene levels in tomato fruits results in increased ABA content and extended shelf-life" (2019). Faculty Publications from the Center for Plant Science Innovation. 220.  
[https://digitalcommons.unl.edu/plantscifacpub/220](https://digitalcommons.unl.edu/plantscifacpub/220)

This Article is brought to you for free and open access by the Plant Science Innovation, Center for at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Faculty Publications from the Center for Plant Science Innovation by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Authors
Gianfranco Diretto, Sarah Frusciante, Claudia Fabbri, Nicolas Schauer, Lucas Busta, Zhonghua Wang, Antonio J. Matas, Alessia Fiore, Jocelyn K. C. Rose, Alisdair R. Fernie, Reinhard Jetter, Benedetta Mattei, Jim Giovannoni, and Giovanni Giuliano
Manipulation of β-carotene levels in tomato fruits results in increased ABA content and extended shelf-life

Gianfranco Direttò a,*, Sarah Frusciante a, Claudia Fabbrì b, Nicolas Schauer c, Lucas Busta d,e, Zhonghua Wang f,g, Antonio J. Matas h,i, Alessia Fiore a, Jocelyn K.C. Rose h, Alisdair R. Fernie e, Reinhard Jetter d,f, Benedetta Mattei b,j, Jim Giovannoni k, and Giovanni Giuliano a,*

a Italian national Agency for New technologies, Energy, and Sustainable Development (ENEA), Casaccia Research Center, 00123 Roma, Italy; b Department of Biology and Biotechnology, Sapienza University of Rome, Italy; c Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, 14476 Potsdam-Golm, Germany; d Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, V6T 1Z4, Canada; e Center for Plant Science Innovation and Department of Biochemistry, University of Nebraska–Lincoln, Lincoln, Nebraska, 68588; f Department of Botany, University of British Columbia, 6270 University Blvd., Vancouver, V6T 1Z4, Canada; g College of Agronomy, Northwest A&F University, Yangling 712100, Shaanxi, China; h Plant Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853, USA; i Institute for Mediterranean and Subtropical Horticulture "La Mayora" (IHSM-UMA-CSIC), Department of Plant Biology, University of Málaga, 29071, Málaga, Spain; j Department of Health, Life and Environmental Sciences, University of L’Aquila, Italy; k U.S. Department of Agriculture–Agricultural Research Service, Robert W. Holley Center for Agriculture and Health, and Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, New York 14853.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pbi.13283

This article is protected by copyright. All rights reserved
corresponding authors:

Tel. +39-06-30483192
Fax +39-06-30483215
E-mail: giovanni.giuliano@enea.it

Tel. +39-06-30483745
Fax +39-06-30483215
E-mail: gianfranco.diretto@enea.it

keywords: tomato, β-carotene, ABA, ripening

Running title: Manipulation of β-carotene levels extends tomato fruit shelf-life
ABSTRACT

Tomato fruit ripening is controlled by the hormone ethylene and by a group of transcription factors, acting upstream of ethylene. During ripening, the linear carotene lycopene accumulates at the expense of cyclic carotenoids. Fruit-specific overexpression of Lycopene β-cyclase (LCYb) resulted in increased β-carotene (provitamin A) content. Unexpectedly, LCYb-overexpressing fruits also exhibited a diverse array of ripening phenotypes, including delayed softening and extended shelf life. These phenotypes were accompanied, at the biochemical level, by an increase of abscisic acid (ABA) content, decreased ethylene production, increased density of cell wall material containing linear pectins with a low degree of methylation, and a thicker cuticle with a higher content of cutin monomers and triterpenoids. The levels of several primary metabolites and phenylpropanoid compounds were also altered in the transgenic fruits, which could be attributed to delayed fruit ripening and/or to ABA. Network correlation analysis and pharmacological experiments with the ABA biosynthesis inhibitor, abamine, indicated that altered ABA levels were a direct effect of the increased β-carotene content and were in turn responsible for the extended shelf life phenotype. Thus, manipulation of β-carotene levels results not only in an improvement of the nutritional value of tomato fruits, but also of their shelf life.
INTRODUCTION

Plants have evolved several mechanisms for seed dispersal, one of which is the development of fleshy fruits with attractive organoleptic characteristics, such as fleshiness, colors and flavors able to attract frugivore animals for seed dispersal. Tomato (*Solanum lycopersicum* L.) is a model system for the study of fruit ripening, mainly due to many genetic and post-genomics resources available for this species (reviewed in (Klee and Giovannoni, 2011) (Seymour et al., 2013) (Gascuel et al., 2017) (Giovannoni et al., 2017)).

Tomato fruit development comprises an initial phase of post-anthesis cell division, followed by one of cell expansion, during which concentrations of the hormones ethylene and abscisic acid (ABA) are both low (Zhang et al., 2009). Immediately, after the mature green (MG) stage of ripening, a transient peak in ABA content occurs, followed by a switch of ethylene production from System 1 (autoinhibitory) to System 2 (autocatalytic), and a peak in ethylene production (Klee and Giovannoni, 2011) (Seymour et al., 2013). Several other events follow, such as fruit softening, the accumulation of sugars and organic acids, and a change of color from green to red, due to the accumulation of the linear carotene, lycopene (Giuliano et al., 1993) (Klee and Giovannoni, 2011). These changes are accompanied, at the molecular level, by extensive changes in gene expression (Alba et al., 2005) (Carbone et al., 2005), with lycopene accumulation being highly associated with the up-regulation of genes encoding *PHYTOENE SYNTHASE 1* (*PSY1*) and *PHYTOENE DESATURASE* (*PDS*) (Giuliano et al., 1993), and the down-regulation of genes encoding *LYCOPENE β- and ε-CYCLASE* (*LCYb* and *LCYe*) (Pecker et al., 1996; Ronen et al., 2000; Ronen et al., 1999). Many of these events have been demonstrated to depend on the presence of a functional ethylene receptor (Alba et al., 2005).

Besides ethylene, tomato fruit ripening is controlled by a cascade of transcription factors, some of which mediate input by other hormones, such as auxin and ABA (Klee and Giovannoni, 2011) (Seymour et al., 2013) (Giovannoni et al., 2017). Exogenous application of ABA at the mature green (MG) stage increases the amplitude of the ethylene peak and
accelerates ripening, while the application of fluridone (a carotenoid biosynthesis inhibitor) or nordihydroguaiaretic acid (an inhibitor of ABA biosynthesis) has the opposite effect (Zhang et al., 2009). Knock-out mutations of \textit{ZEAXANTHIN EPoxidase} (\textit{ZEP}) or silencing of \textit{9-cis-EPOXyCAROTEnOId DIOXYGENase} (\textit{NCED}) result in decreased endogenous ABA and increased ethylene (Galpaz et al., 2008) (Ji et al., 2014), respectively. In contrast, silencing of three \textit{CYP707A2} isoforms, encoding \textit{ABA 8\textquotesingle-hydroxylases} acting in ABA catabolism, or of \textit{ABA uridine diphosphate glucosyltransferase (SlUGT75C1)}, which produces esterified ABA-glucose, generated over-ripe fruits with increased ABA levels (Ji et al., 2014) (Sun et al., 2017). A similar phenotype was observed in RNAi lines for \textit{SlPP2C1}, a \textit{group A type 2C protein phosphatase} involved in ABA signaling (Zhang Y, 2018). These observations suggest that ABA influences fruit ripening in different ways, depending on the mode (external or endogenous) and timing of the application. Since ABA is synthesized from \textit{β-xanthophylls} (Figure 1A), an interesting corollary of the above hypothesis is that carotenoid composition may itself play a role in controlling tomato fruit ripening. At the MG stage, levels of \textit{β-xanthophylls} are high, and during ripening they decline, due to the down-regulation of \textit{LCYb} genes (Pecker et al., 1996; Ronen et al., 2000), possibly affecting ABA levels.

Several reports have described the metabolic engineering of plant carotenoid contents (Giuliano, 2014) (Giuliano, 2017). Overexpression of a \textit{LCYb} gene from Arabidopsis under the control of the ripening-associated \textit{PDS} promoter leads to ripe tomato fruits that accumulate high levels of \textit{β-carotene} (Rosati et al., 2000). Apart from the transgene, these engineered lines are perfectly isogenic with their lycopene-accumulating parental genotype, making them a good system to study the possible influence of carotenoid composition on fruit ripening. Using two independent transgenic lines, we conducted a system-wide study of the effect of increased \textit{β-carotene} levels on tomato fruit ripening and shelf life. Our data suggest that the increase of the \textit{β-carotene} content results in higher ABA content, which in turn has an effect on fruit ripening and shelf life.
RESULTS

*LCYb*-overexpressing fruits exhibit higher β-carotene levels and increased shelf life, which does not correlate with antioxidant activity

Transgenic tomato lines overexpressing Arabidopsis *LCYb* under the control of the chromoplast-associated *PDS* promoter accumulate β-carotene in ripe fruits (Rosati et al., 2000) (Figure 1B-C). Homozygous T4 lines derived from two independent transformation events were grown in the greenhouse, and fruits were harvested at five stages of ripening: Mature Green (MG); Breaker (B); Breaker+4 or pink (B+4), Breaker+10 or ripe (B+10), Breaker+10 or overripe (B+15 and B+40). Expression of *AtLCYb* peaked at B and B+4 (Supplemental Figure 1). A four- to 10-fold increase in β-carotene and other cyclic carotenes and a two- to 3-fold decrease in linear carotenes was observed in B+10 fruits compared to *WT*. Xanthophylls were undetectable in *WT* fruits, but became detectable in *LCYb*-overexpressing fruits (Figure 1C, Supplemental Table 1).

*LCYb*-overexpressing fruits stored at room temperature for several months exhibited, relative to *WT*, a significant increment in shelf life, increased firmness and reduced water loss (Figure 1D). No significant alteration in the time elapsing between anthesis and fruit breaker stage was observed (Supplemental Figure 2), indicating that the alteration was confined to the late stages of fruit development. Since the observed changes in carotenoid levels in *LCYb*-overexpressing fruits are likely to affect fruit antioxidant activity, which is known to impact fruit shelf life (Zhang et al., 2013), we measured the antioxidant activity in both polar and non-polar extracts of *WT* and *LCYb*-overexpressing fruits at five ripening stages (MG, B, B+4, B+10, B+15) (Figure 1D). No significant differences compared to the *WT* were observed at early stages (MG and B). At later stages (B+4 through B+15), *LCYb*-overexpressing fruits displayed a reduction in the antioxidant activity of the non-polar fraction, while that of the polar fraction varied in opposite directions in the two transgenic
lines, with the $LCYb3$ line showing an increase, and the $LCYb1$ line a decrease with respect to the $WT$.

**Increased firmness and decreased water loss of $LCYb$-overexpressing fruits correlate with altered cell wall and cuticle composition**

The firmness of $WT$ and $LCYb$-overexpressing fruits was measured with a hand-held penetrometer at 5 different ripening stages. At MG and B, the firmness of the two types of fruits was similar, then starting at B+4 and until the end of ripening $LCYb$-overexpressing fruits exhibited significantly increased firmness (Figure 2A). One of the components of fruit firmness, evapotranspiration, was found to be significantly lower in $LCYb$-overexpressing fruits (Figure 2B). Total cell wall extracts from the pericarp of B+10 fruits were fractionated by sequential extraction with water, chelating agent, dilute alkali and concentrated alkali (Huisman et al., 1998). Significant increases were observed in the abundance of total cell wall material, water-soluble solids and dilute alkali-soluble solids fractions of $LCYb$-overexpressing fruits. Additionally, the WSS fraction of $LCYb$-overexpressing fruits showed a significant increase in the abundance of galacturonic acid, which is present in the backbone of HG and RG-I polysaccharides (Figure 2C). The occurrence of methyl-esterified pectins in the WSS fraction was analyzed by immunodot analysis using the LM20 monoclonal antibody, which recognizes highly methylesterified HG epitopes. A lower abundance of methyl-esterified pectins was observed in the WSS fraction from $LCYb$-overexpressing fruits, compared to $WT$, while the ChASS fraction did not show significant differences (Figure 2D).

Cuticle thickness was also significantly increased in $LCYb$-overexpressing B+10 fruits (Figure 2E-F). We further investigated the chemical composition of the cutin polymer and the associated cuticular waxes using gas chromatography-mass spectrometry (GC-MS). The amounts of all cutin monomers were significantly higher in the $LCYb$-overexpressing fruits, than in $WT$. Among the cuticular waxes, the major very-long-chain acyl derivatives were
relatively unchanged in \textit{LCYb}-overexpressing fruits, while the triterpenoid components \(\alpha\)-amyrin, taraxasterol, \(\psi\)-taraxasterol and \(\delta\)-amyrin were all > 2-fold greater than \textit{WT} levels (\textit{Supplemental Table 2}).

\textbf{Metabolic remodeling in \textit{LCYb}-overexpressing fruits}

A total of 72 phenylpropanoids were measured in both the flesh and the cuticle of B+10 fruits, using liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) (\textit{Supplemental Table 3}). Most compounds showed an over-accumulation in \textit{LCYb} vs \textit{WT} fruit cuticles. A notable exception was found in the group of phenolic compounds, such as 1-caffeoyl-1-beta-D-glucose, 4-\(p\)-coumaroylquinic acid, chlorogenic, coumaric, dicaffeoylquinic acid and ferulic acids, which showed a slight decrease in the flesh of \textit{LCYb}-overexpressing fruits relative to \textit{WT}; on the contrary, stronger positive variations were observed for flavonoids and flavonoid glycosides: 26 out of 48 metabolites showed significantly higher levels in \textit{LCYb}-overexpressing fruits, with catechin/epicatechin, eriodictyol, kaempferol and quercetin conjugates showing the largest increases (\textit{Supplemental Table 3}).

Additionally, the levels of 58 metabolites (20 amino acids, 19 sugars/polyols, eight organic acids and 11 others) were quantified by GC-MS in ripe fruits of two \textit{LCYb}-overexpressing lines (Schauer et al., 2005) (\textit{Supplemental Table 4}). Thirteen compounds (five amino acids, seven sugars/polyols, and putrescine) showed significant changes in both transgenic lines. The amino acids alanine, aspartate, phenylalanine and proline, the sugars mannitol and sucrose, the organic acid 2-oxo-butyric acid and the polyamine putrescine were lower in \textit{LCYb}-overexpressing fruits. In contrast, the sugars erythritol, galactinol, glucose, glucoheptose, melibiose, and the sugar phosphate fructose 6-P were higher. When averaged between the two \textit{LCYb} lines, all changes were less than 2-fold in magnitude (\textit{Supplemental Table 4}), suggesting that changes in primary metabolism were minor.
LCYb-overexpressing fruits exhibit increased ABA and decreased ethylene production and altered expression of the RIN and NOR ripening regulators

ABA is synthesized from violaxanthin and neoxanthin, which are increased in LCYb-overexpressing fruits (Figure 1A-B). ABA accumulation during ripening was measured using LC-HRMS (Figure 3A, Supplemental Table 5) and showed very distinct kinetics in WT and LCYb-overexpressing fruits: in WT fruits, ABA levels peaked at the B stage, and then progressively declined until B+15, while in LCYb-overexpressing fruits they showed a much larger peak at the B stage, followed by a further increase to 17-fold WT levels at B+15. ABA catabolites (phaseic acid, dihydrophaseic acid, ABA-Glc and 7-hydroxy-ABA) also showed increased levels in LCYb-overexpressing fruits (Supplemental Table 5). These data indicate that LCYb overexpression during ripening causes accumulation of β-carotene and β-xanthophylls, and that the increased flux through the β-branch of carotene biosynthesis results in increased levels of ABA and its downstream catabolites.

We also measured ethylene emission by intact fruits, and the enzymatic activity of ACC oxidase, the last enzyme in the ethylene biosynthetic pathway. Ethylene production and ACC oxidase activity both peaked at B+4 in WT fruits, and the peak showed an approximately 50% reduction in LCYb-overexpressing fruits relative to WT (Figure 3B-C).

The kinetics of expression of two key regulators of fruit ripening, RIN (Vrebalov et al., 2002) and NAC-NOR (Giovannoni, 2004) (Osorio et al., 2011), were analyzed by quantitative real-time RT-PCR (qRT-PCR) in WT and LCYb-overexpressing fruits (Figure 3D-E). The RIN transcript was strongly repressed starting at the B stage and throughout the whole ripening process, while, on the contrary, NAC-NOR displayed a large increase in expression at the B stage.

Systems analysis of WT and LCYb-overexpressing fruits
Transcript profiling was performed on fruits at three ripening stages (MG, B and B+10) in the WT and two LCYb-overexpressing lines using the EU-TOM3 Affymetrix microarray (Supplemental Tables 6-8-10A/B). Genes up- or down-regulated >1.5-fold in both transgenic lines with respect to WT, with a P-value ≤0.05 were considered differentially regulated and are shown as Mapman representations in Supplemental Figure 3. A total of 123 transcripts were found to be differentially regulated in both the MG and B stages, 137 in both B and B+10, and 148 in both MG and B+10 (Supplemental Figure 4, Supplemental Table 12). GO enrichment analysis (Supplemental Tables 7-9-11A/B) showed a series of enriched GO terms in up-regulated genes (tetrapyrrole/chlorophyll and protein binding and amine metabolism at the MG and B+10 stages, respectively), and in down-regulated ones (carbohydrate and nucleotide metabolism). We also performed a manual annotation of differentially regulated transcripts involved in well-known aspects of fruit ripening, including ethylene metabolism and regulation, cell wall remodeling, cuticle biogenesis, primary metabolism, phenylpropanoid, carotenoid and apocarotenoid pathways (including ABA). All of the aforementioned classes were represented in differentially regulated genes, with ethylene- and cell wall-related genes showing the highest number of differentially expressed (particularly down-regulated) representatives (Supplemental Tables 6-8-10A/B). Interestingly, a series of key genes in the phenylpropanoid pathway (PHENYLALANINE AMMONIA-LYASE (PAL) and CHALCONE SYNTHASE (CHS) at the MG stage; PAL and 4-COUMARATE:COA LIGASE 2 (4CL2) at the B stage; CHS and DIHYDROFLAVONOL 4-REDUCTASE (DFR) at the B+10 stage) were also overexpressed in LCYb-overexpressing fruits compared to the WT.

The levels of 28 additional transcripts involved in fruit ripening control were measured in B+10 fruits using through qRT-PCR (Supplemental Table 13). The majority of transcription factors, including RIN, TAGL1 (Vrebalov et al., 2009) (Itkin et al., 2009), CNR (Manning et al., 2006), HB-1 (Lin et al., 2008), AP2a (Chung et al., 2010) (Karlova et al., 2011) and TDR4/FUL1 (Bemer et al., 2012) were down-regulated in LCYb-overexpressing fruits. Down-regulated transcripts also included the NEVER-RIPE ethylene
receptor, GR, CTR1 and EIN2, participating in ethylene signaling (Barry et al., 1996) (Fu et al., 2005), and ACS2, ACS4 and ACO1 genes (Bidonde et al., 1998) (Barry et al., 2000), involved in ethylene biosynthesis. Notable exceptions to this pattern were the NAC-NOR transcription factor (Giovannoni, 2004) (Osorio et al., 2011), and the E4 and E8 ethylene-inducible genes (Cordes et al., 1989), which were up-regulated in LCYb-overexpressing fruits. Several genes involved in cell wall degradation/remodeling were also down-regulated, such as POLYGALACTURONASE 1 (PG1), PECTATE LYASE (PL), α-L-ARABINOFURANOSIDASE 1 (ARF1), EXPANSIN 1 (EXP1), β-D-XYLOSIDASE 1 and 2 (XYL1, XYL2), and XYLOGLUCAN ENDO-TRANSGLUCOSYLASE/HYDROLASE 4 and 8 (SIXTH4, SIXTH8) genes. Exceptions included the PECTIN METHYLESTERASE 1 and 2 (PMEU1, PMEU2) genes, which were up-regulated, consistent with the decreased levels of pectin methyl-esterification noted above.

The metabolic and transcriptional alterations observed in LCYb-overexpressing fruits at B+10, associated with ABA, ethylene, cuticle and cell wall metabolism are summarized as ad hoc Mapman charts (Thimm et al., 2004) in Figure 4. LCYb overexpression in fruits resulted in extensive perturbations of ABA and ethylene metabolism and signal transduction, and also of cuticle and cell wall biogenesis.

In order to identify co-regulated traits (e.g. metabolites, hormones, transcripts, phenotypic traits), we chose 1,612 variables that are differentially regulated in LCYb-overexpressing with respect to WT fruits. The Pearson correlation coefficient values (ρ) for the resulting trait pairs (Supplemental Table 14) were used to build a correlation network (Diretto et al., 2010a), including correlations |ρ| > 0.90 (Figure 5, Supplemental Table 15). The overall “network strength” (i.e., the average of all |ρ| values (Diretto et al., 2010a) was very high (0.97), indicating that the variables are tightly co-regulated. Several metabolites, hormones and phenotypic traits associated with fruit ripening grouped as a tight cluster, in a region populated by known ripening regulators. Notably, ABA had a central position in this network and was strongly co-regulated with NAC-NOR (ρ=0.99), RIN (ρ=-0.99), and AP2a
(\(\rho = -0.99\)), and less so with ethylene, \(NR, HB-I, CNR\) and \(TAGLI\). \(NAC-NOR, RIN\) and \(AP2a\) were also strongly co-regulated with ethylene (\(\rho = 0.96, 0.98\) and 0.97, respectively). Interestingly, the \(CNR\) ripening regulator (Manning et al., 2006) was more strongly co-regulated with ABA (-0.90) than with ethylene (0.73). We also constructed sub-networks centered around ABA and ethylene. The ABA network (Supplemental Figure 5A, Supplemental Table 16A) included the vast majority of transcription factor genes known to control fruit ripening: \(NAC-NOR, TDR4, AP2A, HB-I, RIN, TAGLI\) and \(CNR\). Of these, \(NAC-NOR\) showed positive co-regulation with ABA, while all other genes showed negative co-regulation (Supplemental Tables 15-16A). Additional genes strongly co-regulated with ABA included genes involved in ABA signal transduction, genes for ethylene biosynthesis, sensing and signal transduction, and genes for carotenoid, chlorophyll and cell wall metabolism (Supplemental Table 16A). In the second network, centered around ethylene (Supplemental Figure 5B; Supplemental Table 16B), strongly co-regulated genes were involved in ethylene biosynthesis, sensing and signal transduction, but also key ripening regulators and genes involved in ABA signal transduction (Supplemental Tables 15-16B). This is consistent with recent suggestions of substantial cross-talk between the networks controlling these hormones during ripening (Galpaz et al., 2008) (Ji et al., 2014) (Sun et al., 2017).

**Abamine treatment of \(LCYb\)-overexpressing fruits reduces ABA accumulation, increases ethylene production, and reverses the long shelf life phenotype**

Abamine is a well-known inhibitor of NCED enzymatic action and of ABA biosynthesis (Han et al., 2004). To better investigate the ABA role in the extended shelf-life phenotype, we treated \(LCYb\)-overexpressing fruits with abamine at the MG stage. The treatment resulted in a reduction of ABA in \(LCYb\)-overexpressing fruits to levels similar to \(WT\) ones (Figure 6A). As a result, ethylene production was increased to levels slightly higher than those of \(WT\) fruits (probably as a result of the injection), while flesh firmness and water loss reverted to \(WT\) levels (Figure 6B-D).
DISCUSSION

LCYb overexpression in tomato fruits resulted in increased β-carotene content and fruit firmness and in extended shelf life. At the biochemical level, this phenotype was accompanied by a modification of cell wall composition and polymerization, of cuticle thickness and chemical composition, of primary metabolite and phenylpropanoid profiles in the fruit pericarp. This is, to our knowledge, the first time that metabolic engineering of carotenoid biosynthesis has been reported to have such profound and pleiotropic effects on fruit ripening. Until now, carotenoid composition has merely been considered as an output of the regulatory circuit controlling fruit ripening.

Metabolic alterations in LCYb-overexpressing fruits

Some of the metabolic perturbations observed in LCYb-overexpressing fruits can be attributed to their delayed ripening: compounds like alanine, aspartate, proline, mannitol, and putrescine increase during normal fruit ripening (Carrari et al., 2006) and show a decrease in ripe LCYb-overexpressing fruits compared to WT ones. Other metabolic perturbations can be attributed to the observed changes in gene expression: for instance, the decrease in sucrose and increase in glucose levels correspond to an induction, at the B stage, of ACID INVERTASE, which hydrolyzes sucrose into glucose and fructose. The functional role of acid invertase is very well characterized in tomato fruits, with quantitative trait loci, genome wide association studies and reverse genetic approaches, all indicating its importance for determining soluble solids content (Fridman et al., 2004) (Tieman, 2017) and aspects of fruit development and seed yield (Zanor et al., 2009). Also, overexpression of ACID INVERTASE has been observed in tomato fruits overexpressing an ABA-response element binding factor (SIAREB1) which leaves ethylene levels unaltered (Bastias et al., 2011), indicating that this trait may be under direct ABA control. Interestingly, LCYb-overexpressing fruits share considerable commonalities with those of
the well characterized ripening mutants *rin, nor, NR* and *ap2* (Osorio et al., 2011) (Karlova et al., 2011) with decreases in alanine, aspartic acid, glutamic acid, glutamine, phenylalanine and proline all being observed in *rin*, *nor* and *NR* and the changes in aspartic acid, glutamine, phenylalanine and proline also being observed in *ap2*. Glucose also showed consistent trends between ripening mutants and *LCYb*-overexpressing fruits, being over-accumulated in all the genotypes; while sucrose, present at higher contents in *nor, rin, NR*, displayed an opposite trend, i.e. reduction, in *LCYb*-overexpressing fruits.

Additional metabolic fluctuations that can be attributed to ABA accumulation are the increase in flavonoids. The extent of changes (>10 fold for some flavonoid glycosides) suggests they represent direct effects of the genetic manipulation, and thus of the increase in ABA in *LCYb*-overexpressing fruits, compared to the less pronounced alterations observed in primary metabolites, which are likely to represent secondary effects. This hypothesis finds support in a series of previous studies linking ABA and flavonoid levels in apple (Lu et al., 2017), soybean (Gupta et al., 2018) and tomato (Mou et al., 2015). In agreement with biochemical data, a series of key structural phenylpropanoid genes were up-regulated in *LCYb*-overexpressing fruits: for instance, *PHENYLALANINE AMMONIA-LYASE (PAL)* at the MG and B stages, and *CHALCONE SYNTHASE (CHS)* at the MG and B+10 stages. ABA has been shown previously to promote *PAL* and *CHS* expression (Zhang et al., 2017); (Yu et al., 2015), and PAL activity (Jiang and Joyce, 2003).

Purple tomatoes, overexpressing *Del* and *Ros* transcription factors from snapdragon and accumulating large amounts of anthocyanins display extended shelf life, a phenotype attributed to the increased total antioxidant activity caused by anthocyanin accumulation (Zhang et al., 2013). While we cannot exclude that the increase of flavonoid levels in the peel of *LCYb*-overexpressing fruits influences its permeability and hence, water loss, total antioxidant activity does not seem to have a causal relationship with the extended shelf-life of these fruits: the fluctuations of total antioxidant activity in the polar fraction did not correlate with fruit shelf life, while those in the non-polar fraction showed a negative
correlation. This finding is not surprising, since β-carotene has been reported to have a lower antioxidant activity compared to lycopene (Bohm and Schwartz, 2002)). Thus, the conversion of large part of lycopene into β-carotene in LCYb-overexpressing fruits is consistent with the observed decrease of anti-oxidant activity in the non-polar fraction.

**Alterations in cell wall and cuticle composition**

The extraction and subsequent fractionation of the cell walls of LCYb-overexpressing fruits yielded higher amounts of total cell walls, water-soluble and diluted alkali-soluble solids per unit weight compared to WT, indicating alterations both in the content and in the solubility of cell wall polymers. The higher content of the pectic backbone sugar GalA in the WSS fraction suggested a higher content of pectins, which were less methyl-esterified than those from WT fruits. The regulated swelling and disassembly of primary cell walls and the modification of the middle lamellae between adherent primary cell walls are thought to be important factors contributing to tissue softening during tomato fruit ripening. The increased abundance in LCYb-overexpressing fruits of linear, low-esterified homogalacturonan is likely to influence both cell adhesion and fruit texture. Pectins secreted to the cell wall possess methyl-ester side chains, which are removed by pectin methylesterase (PME) as a prerequisite for polygalacturonase (PG) action (Wakabayashi et al., 2003). PME can play dual and contrasting roles within the plant cell wall: on one hand, it generates blocks of de-esterified galacturonic acid residues within the pectin polymer that can be cross-linked by calcium, thus strengthening the cell-to-cell adhesion; on the other hand, the same de-methyl-esterified blocks may be more susceptible to degradation by PG. While PG-mediated polyuronide depolymerization during ripening does not appear to be the primary determinant of tomato fruit softening (Giovannoni, 1989) (Brummell, 2001) (Uluisik et al., 2016), experimental data suggest a role for PMEs in determining fruit firmness: silencing of PMEU1 resulted in faster softening during fruit ripening (Phan et al., 2007), while silencing PMEU2 results in loss of tissue integrity during fruit senescence.
Additional genes contribute to tomato fruit softening during ripening: EXP1 and PL silencing resulted in a moderate increase in fruit firmness throughout ripening (Brummell et al., 1999) (Uluisik et al., 2016) (Wang et al., 2019). The expression of genes involved in cell wall remodeling in LCYb-overexpressing fruits is consistent with their phenotypes: PL, PG and EXP1 are down-regulated while PMEU1/2 are up-regulated compared to WT. Taken together, these results indicate that LCYb overexpression affects the ripening-associated pathway leading to pectin solubilization and cell wall disassembly.

LCYb-overexpressing fruits also had significantly higher amounts of cutin monomers and alicyclic wax components relative to WT, but unchanged levels of linear, very-long-chain wax compounds. Our results suggest a regulatory effect of ABA on cuticle deposition in fruits, consistent with previously published data in tomato (Curvers et al., 2010) (Martin et al., 2017) and Arabidopsis (Seo et al., 2011) (Zhang et al., 2005) leaves.

Cross-talk between carotenoids, ABA, and ethylene in the control of fruit ripening

Carotenoids, and more specifically 9-cis-epoxyxanthophylls, synthesized from β-carotene, are metabolic precursors of ABA (Figure 1A) (Qin and Zeevaart, 1999) (Giuliano et al., 2003). The increase in ABA levels observed in LCYb-overexpressing fruits is an indirect consequence of the increase in the β-carotene pool. This is, to some extent, unexpected: the rate-limiting step for ABA biosynthesis in leaves is believed to be the cleavage of 9-cis-epoxyxanthophylls by the NCED dioxygenase (Qin and Zeevaart, 1999); (Thompson et al., 2000); (Giuliano et al., 2003). However, the level of β-carotene in WT tomato fruits is only 1.37-fold higher than that of ABA and β-carotene and ABA levels are strictly co-regulated in LCYb-overexpressing fruits (Supplemental Tables 16-17), suggesting that in tomato fruits β-cyclization is rate-limiting for ABA biosynthesis. Since the β-cyclization step is itself regulated during ripening (Pecker et al., 1996), this has important implications for the regulatory circuits controlling tomato fruit ripening (see below).
Cross-talk between the ABA and ethylene signaling pathways has been described in Arabidopsis, where a screen for extragenic enhancers or suppressors of ABA-insensitive \( abi1 \) mutant resulted in alleles of the constitutive ethylene response mutant \( ctr1 \) and ethylene-insensitive mutant \( ein2 \) (Beaudoin et al., 2000). Blocking of ethylene biosynthesis before véraison in grape, a non-climacteric fruit, results in inhibition of ABA biosynthesis and of fruit ripening (Sun et al., 2010). This indicates that the cross-talk between ABA and ethylene is probably present in different species and in both climacteric and non-climacteric fruits. Both positive and negative cross-talk have been reported in tomato fruits between ABA and ethylene, depending on the time and mode of application of the former hormone: exogenous application of ABA during early fruit development induces fruit ethylene biosynthesis and accelerates ripening in a fashion dependent on the \( RIN \) ripening regulator (Mizrahi et al., 1975) (Zhang et al., 2009); exogenous application of ABA or nordihydroguaiaretic acid, an inhibitor of ABA synthesis, respectively, accelerated or delayed fruit ripening, with a simultaneous higher and lower emission in ethylene (Zhang et al., 2009). Furthermore, reduced endogenous ABA levels in fruits of the tomato \( hp3 \) mutant resulted in increased ethylene production (Galpaz et al., 2008). Cross-talk of ABA and ethylene during in tomato fruit ripening has also been observed in fruits with altered expression of transcription factors like \( NAC1 \) (Meng et al., 2016) and \( ZFP2 \) (Weng et al., 2015).

In \( LCYb \)-overexpressing fruits, the increase in ABA levels results in a corresponding decrease of the ethylene peak, a phenotype exactly symmetric to that of the \( hp3 \) mutant (Galpaz et al., 2008). These data supporting a negative cross-talk between the two hormones. How does this negative cross-talk occur in \( LCYb \)-overexpressing fruits? Our data indicate that ABA levels are strongly co-regulated with transcript levels of several ripening regulators, including \( NAC-NOR \) and \( RIN \) (Supplemental Table 15-16A). \( RIN \) (Vrebalov et al., 2002) encodes a MADS box transcription factor (Ito et al., 2017) (Li et al., 2017), and \( NAC-NOR \) (Giovannoni, 2004) belongs to the NAC domain family of
transcription factors, many of which are involved in responses to ABA (Nakashima et al., 2012). Both NAC-NOR and RIN are necessary for ethylene synthesis and fruit ripening. NAC-NOR acts upstream of RIN (Tigchelaar, 1973), and RIN binds promoter elements of ripening-associated genes in a CNR-dependent fashion (Fujisawa et al., 2013) (Martel et al., 2011). Increased PG gene expression and increased susceptibility of ripe fruits to Botrytis cinerea requires NOR, but not RIN (Dellapenna et al., 1987) (Cantu et al., 2009), while induction during ripening of transcripts involved in ethylene biosynthesis is completely abolished in nor, but only partially in rin fruits (Osorio et al., 2011). ABA accumulation in rin fruits is similar to WT fruits, while in nor fruits it is decreased (McGlasson and Adato, 1976). Two recent studies suggested strong regulatory relationships between NOR and ABA: SlAREBI, a transcription factor involved in ABA signaling, directly regulates NOR expression (Mou et al., 2018) and tomato de penjar-type cultivars, characterized by extended shelf-life, displayed a nor mutation, increased ABA levels and reduced water loss (Kumar et al., 2018). An interesting hypothesis is that ABA could regulate directly the expression of NAC-NOR and/or RIN. A series of ABA-response elements such as ABRE, DRE, LTRE, MYB and MYC are localized in the promoter regions of NOR and RIN (Supplemental Table 18).

Additional mutants are known to affect β-carotene biosynthesis in tomato fruits, such as old gold and Beta, which result respectively in an impairment or an enhancement, of the fruit-specific CYC-b cyclase (Ronen et al., 2000). An old gold allele shows increased fruit firmness, although its fruit ABA content has not been studied (Silletti et al., 2013). Beta alleles carry a poorly characterized chromosomal introgression from green-fruit species, which results in complex vegetative and fruit phenotypes (unpublished data). Additional ABA biosynthesis mutants, like flacca and sitiens, exhibit a wilty phenotype and reduced plant and fruit size (Nitsch et al., 2012). These characteristics of the mutants, the fact that the mutations are expressed throughout vegetative growth and fruit ripening, and the hypothesized dual role of ABA in regulating ripening (see below), complicate the interpretation of the mutant phenotypes.
A model to explain the phenotypic alterations of LCYb-overexpressing fruits

Based on the data gathered, we propose a model to explain the phenotypes observed in LCYb-fruits. First, β-carotene levels appear to be the main driver of ABA levels during late ripening, as suggested by the parallel increase of the two metabolites in LCYb-overexpressing fruits. Second, while at the MG stage ABA acts as a trigger for ripening (Zhang et al., 2009), during late ripening it turns into a negative regulator of both ethylene production and fruit ripening (this paper). This dual role of ABA in regulating tomato fruit ripening is supported by the results obtained with RNAi lines where NCED silencing is driven by the fruit-specific E8 promoter: these lines show decreased ABA and increased ethylene levels during late ripening (Sun et al., 2012). A dual role of ABA in regulating fruit ripening has been also described in peach, where its application at the S3 stage represses ripening, while application at the S4 instead accelerates it (Soto et al., 2013).

In our model (Figure 7), primary effects include those known to be under direct ABA control, such as phenylpropanoid content, cuticle thickness, cutin and triterpenoid composition; secondary effects include instead those not directly associated to ABA, such as cell wall and primary metabolite composition. Since β-carotene in fruits is mainly synthesized as a result of CYC-B activity (Ronen et al., 2000), and CYC-B expression is negatively affected by ethylene (Alba et al., 2005), we hypothesize that a negative feedback loop, involving CYC-B, β-carotene, ABA and ethylene, is active during late fruit ripening, in which ethylene represses CYC-B, lowering the levels of ABA and thus enhancing its own levels and accelerating ripening. In LCYb-overexpressing fruits, lycopene β-cyclace activity and β-carotene levels are no more under negative ethylene control, and thus ABA levels remain high during late ripening, repressing ethylene production and extending shelf life.

Our model is strongly supported by the pharmacological experiment shown in Figure 6: abamine, a known inhibitor of NCED activity, decreases ABA levels in ripe LCYb-
overexpressing fruits, and simultaneously decreases fruit firmness and water loss and increases ethylene production. This is a strong suggestion that β-carotene exerts its effects on fruit ripening through its cleavage product, ABA, and not through the alteration of the fruit antioxidant potential, as in the case of the Del/Ros tomatoes. Additional studies with inhibitors of ABA synthesis and sensing will shed more light on the role of this hormone in the control of tomato fruit ripening.
METHODS

Plant material and fruit sampling

The LCYb transgenic tomato plants have been previously described (Rosati et al., 2000). Growth of plants was as previously described (Giliberto et al., 2005). Time to Breaker was measured by tagging flowers at anthesis. Fruits were harvested at five ripening stages (mature green, MG; breaker, B; 4, 10 and 15 days after the breaker stage, B+4, B+10 and B+15); at least six fruits from three different plants (three biological replicates) were harvested, cut in small pieces, and frozen in liquid nitrogen. Pooled fruits for each biological replicate were stored at -80°C for a maximum of 6 months before biochemical and transcriptomic analyses.

Analysis of ABA content

Frozen pericarp tissues at 5 ripening stages were lyophilized and ground to a fine powder. At least three different pericarp pools (biological replicates) were analyzed for each genotype. 200 mg were extracted for each replicate as previously described (Welsch et al., 2008). LC-HRMS was carried out using a Finnigan Surveyor Plus HPLC system (Thermo Electron), equipped with a 3μm Hypersil Gold C18 reverse-phase column (150 X 4.6 mm; Thermo Electron) as reported before (Ross et al., 2004). Internal standard-based quantification was carried out using the MS data and the quantification software available in the Xcalibur 2.0 software package. Retention times and MS² fragmentation patterns were used for identification by using authentic reference standards (trans-ABA from OlChemIm and (±)-ABA from Sigma).

Biochemical and phenotypic assays

Ethylene production was measured on freshly harvested fruits as described (Thompson et al., 1999). At least 10 fruits, with uniform size and pigmentation were analyzed for each line and developmental stage using a Carlo Erba Fractovap 4200 gas chromatograph (Carlo Erba Spa, Milan, Italy) equipped with a flame ionization detector (FID) and 1-m-long alumina
column (80-100 mesh) at 80°C. A calibration curve was performed using known concentrations of ethylene. ACO enzymatic activity was assayed according to the protocol from (Barry et al., 1996). Water loss was measured, for each genotype, on at least 10 fruits from 3 different plants, kept at constant temperature and relative humidity (22±2°C, 60±5%). Fruit firmness was evaluated using two different mechanical tests and at least 10 fruits for each line. Pulp firmness was checked on peeled fruits with a 1 kg hand-held fruit pressure tester (Turon, Cesena, Italy) equipped with an 8-mm probe. Pericarp thickness was measured using a caliper. Cuticle isolation was performed on fruits at B+10 as previously described (Saladie et al., 2006). At least four strips/fruit were included in paraffin and fixed in formaldehyde:acetic acid 1:1 (v/v) in 18 volumes of 70% alcohol for 48 h, followed by dehydration in an alcohol series (50-10%) and a water wash. At least 4 sections of 10 μm per fruit were cut with a microtome and stained with 0.05% of Toluidine Blue. Cuticle thickness was determined using light microscope images (40x magnification) and the ImageJ image analysis software (http://rsb.info.nih.gov/ij).

**Cell wall fractionation and composition analysis**

Preparation and extraction of total cell wall (TCW) was carried out essentially as described in (Orfila et al., 2002). Four fractions were extracted sequentially from TCW as reported (Huisman et al., 1998). Monosaccharide composition was determined by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Ion Chromatography System, ICS 3000, Dionex, CA, USA) as described (Lionetti et al., 2010). The column was a CarboPac PA20 column 3 x 150 mm (Dionex, CA, USA), equipped with a CarboPac PA20 guard column 3 x 30 mm. Peaks were identified and quantified by comparison to a standard mixture of fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid (Sigma, St. Louis, MO, USA). All data are expressed as mean ± S.D. Specific pectic epitopes were detected by immunodot assay with LM20 antibody obtained from PlantProbes (UK). Polysaccharide solutions (5 mg/ml) in 0.5% (w/v) ammonium oxalate buffer were spotted as 1 μl drops onto nitrocellulose membrane (Biorad) in a 3-fold dilution series. Membranes were allowed to air dry at room
temperature for 1 h, then blocked with 3% blocking reagent (GE Healthcare) in phosphate-buffered saline (PBS; Bio-Rad) for 1 h prior to incubation for 1.5 h with LM20. After extensive washes in PBS, membranes were incubated with anti-rat secondary antibody conjugated to horseradish peroxidase (GE Healthcare) and washed in PBS prior to detection with ECL detection reagent (GE Healthcare). For each analysis, at least three biological replicates were performed.

Cuticle composition analysis

Cuticular waxes and cutin were analyzed as previously described (Yeats et al., 2012). Samples were separated by gas chromatography (5890 II, Hewlett Packard, Avondale, PA, USA; 30 m DB-1, 0.32 mm i.d., df=1 μm, J&W Scientific, Folsom, CA, USA) with He carrier gas (1.4 ml/min) and mass spectrometric detection (MS; 5971N, Agilent, EI, 70 eV, m/z 50–800, 1 scan per sec.). For analyte quantification, an identical GC system was used, except that a flame ionization detector (FID) was used, which burned H2 (30 ml/min) in air (200 ml/min), and used N2 to shape the flame (20 ml/min). Analytes were quantified against the internal standard by manual integration of peak areas. For each analysis, at least three biological replicates were performed.

qRT-PCR and microarray analyses

RNA isolation and Real Time qRT-PCR conditions were as previously reported (Diretto et al., 2010b). List of primers for each gene is reported in Supplemental Table 10. Normalization to a housekeeping gene (actin) and to WT values were applied to raw data to obtain relative expression levels. For each genotype, at least three biological replicates were performed.

Microarray experiments were carried out using GeneChip® EU-TOM3 platform (Affymetrix) and an external service provided by IFOM (Fondazione Istituto FIRC di Oncologia Molecolare - COGENTECH, Milan, Italy) as previously described (Mori et al., 2012). CEL files were subsequently analyzed with RobiNA software (Lohse et al., 2012). Briefly, subsequent steps of quality assessment, data normalization and identification of
genes differentially regulated between WT and LCYb-overexpressing lines (LCYb1 and LCYb3) fruits for each ripening stage were carried out. The raw data were then normalized using the RMA method. Statistical analysis of pair-wise differential gene expression were performed using a linear model-based approach, applying a 0.05 cut-off for \( P \)-values after a False Discovery Rate (FDR) correction. Microarray experiments have been deposited to the GEO public repository (https://www.ncbi.nlm.nih.gov/geo) under the accession number GSE108415. For each genotype, at least three biological replicates were performed.

**Primary metabolite analyses**

Metabolite analysis of ripe (B+10) tomato peeled pericarp samples (300 mg) by GC-MS was carried out essentially as described in (Lisec et al., 2006), with specific modifications for tomato tissues as described in (Schauer et al., 2006). The GC-MS system used comprised an AS 2000 autosampler, a GC 8000 gas chromatograph and a Voyager quadrupole mass spectrometer (ThermoFinnigan, Manchester, UK). The mass spectrometer was tuned according to the manufacturer’s recommendations using tris-(perfluorobutyl)-amine (CF43). Both chromatograms and mass spectra were evaluated using the MASSLAB program (ThermoQuest, Manchester, UK) with reference to libraries of the Golm Metabolite Database (Kopka et al., 2005) (Schauer et al., 2005). For each genotype, at least 3 biological replicates were performed.

**Non polar and semi-polar metabolite analyses**

Non polar (carotenoids) and semi-polar (phenylpropanoid) analyses were carried out by liquid chromatography coupled to diode-array detector and Atmospheric pressure chemical ionization- high resolution mass spectrometry (LC-DAD-APCI-HRMS) or Electrospray ionization (LC-DAD-ESI-HRMS), respectively, operating in positive and negative ion modes, as previously described (Su et al., 2015); (Fasano et al., 2016); (D'Esposito et al., 2017)). Identification of carotenoids was performed as reported previously (Liu et al., 2014). Phenylpropanoid analysis was performed by comparing chromatographic and spectral properties with standards and reference DAD-HRMS spectra as previously reported (Moco
et al., 2006); (Iijima et al., 2008); (Fernandez-Moreno et al., 2016). For each analysis, at least three biological replicates were performed.

**Total antioxidant capacity**
Fruit total antioxidant capacity was determined as previously described (Enfissi et al., 2010), by estimating the capacity of non polar extract to quench the ABTS⁺ radical via measuring Abs at 734 nm compared to the one of Trolox. Results were expressed as a TEAC in mM of Trolox per gram of DW. For each line, at least three biological replicates were performed.

**Abamine-pharmacological treatment**
Abamine-treatment of LCYb-overexpressing fruits was carried out as previously described (Mou et al., 2016); (Su et al., 2015); (Brandi et al., 2011) using, for each fruit, 1 ml of 1 mM abamine, dissolved in dimethylsulfoxide (DMSO). At least 5 MG fruits for each genotype were collected and subjected to treatment with abamine or DMSO (mock). ABA, fruit firmness, ethylene emission and water loss were evaluated as previously described.

**Statistical and bioinformatic analyses**
The significance of differences between WT and LCYb-overexpressing fruits was evaluated using Student’s t-test (*P < 0.05, **P < 0.01). For an easier homogenization and interpretation of the results, all data were normalized on the WT values. When the levels of a metabolite or gene expression were “not detectable”, an arbitrary value was set, corresponding to 1/10 of the lowest value in the dataset. Normalized data were log2 transformed and visualized on public and ad hoc metabolic maps using the MapMan software (Urbanczyk-Wochniak et al., 2006). For enrichment analysis, Solyc of genes up- or down-regulated were subjected to Gene Ontology Enrichment Analysis (GOEA) using the Plant MetGenMap tool (http://bioinfo.bti.cornell.edu/tool/GO/GO_enrich.html; (Joung et al., 2009)). Overrepresented GO terms in each category (biological process, molecular function, cellular component) were determined using the False Discovery Rate (FDR) statistical method and a p value ≤ 0.05 (Benjamini, 2001). Correlation networks were generated by
Cytoscape version 2.6.3 (www.cytoscape.org; (Cline, 2007)), as previously described ((Rambla et al., 2016); (Aversano et al., 2017)), and visualized as force-directed layouts weighted with \( \log_2 (1-|\rho|) \) values.

ACKNOWLEDGMENTS

The work was supported by the European Commission (Projects DISCO, grant agreement: 613513; and Traditom, grant agreement: 634561) to GG, a Special Research Opportunity grant from the Natural Sciences and Engineering Research Council of Canada, the Canada Chairs Program and the Canada Foundation for Innovation to RJ and the United States Department of Agriculture – Agricultural Research Service and the US National Science Foundation (grant IOS-1539831) to JG, and benefited from the networking activities within the European Cooperation in Science and Technology Action FA1106 (Qualityfruit) and CA15136 (EUROCAROTEN). We thank Florian Wurst and Peter Beyer, Freiburg University (Germany), for help in ABA measurements; Asaph Aharoni and Yoseph Hirschberg for providing annotated tomato cuticle and ABA-relative gene lists, respectively; and Christian Chervin, Julien Pirrello and Mondher Boutzayen for help in fruit abamine-treatments. The authors declare to have no conflict of interest.

AUTHOR CONTRIBUTIONS

GG, JG and GD designed the research. GD and AF selected independent transgenic lines. GD and SF performed phenotypic, microarray/qRT-PCR analyses, and carotenoid/phenylpropanoid metabolomic analyses. CF and BM carried out cell wall analyses. NS and ARF performed primary metabolite determinations. ZW, LB and RJ carried out cuticle metabolomics. AJM and JKCR performed cuticle microscopy. All authors analyzed data, wrote and agreed the final version of the paper.
REFERENCES

Alba, R., Payton, P., Fei, Z., McQuinn, R., Debbie, P., Martin, G.B., Tanksley, S.D. and Giovannoni, J.J. (2005) Transcriptome and Selected Metabolite Analyses Reveal Multiple Points of Ethylene Control during Tomato Fruit Development. Plant Cell 17, 2954-2965.

Aversano, R., Contaldi, F., Adelfi, M.G., D'Amelia, V., Diretto, G., De Tommasi, N., Vaccaro, C., Vassallo, A. and Carputo, D. (2017) Comparative metabolite and genome analysis of tuber-bearing potato species. Phytochemistry 137, 42-51.

Barry, C.S., Blume, B., Bouzayen, M., Cooper, W., Hamilton, A.J. and Grier, D. (1996) Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant J 9, 525-535.

Barry, C.S., Llop-Tous, M.I. and Grier, D. (2000) The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. Plant Physiol 123, 979-986.

Bastias, A., Lopez-Climent, M., Valcarcel, M., Rosello, S., Gomez-Cadenas, A. and Casaretto, J.A. (2011) Modulation of organic acids and sugar content in tomato fruits by an abscisic acid-regulated transcription factor. Physiol Plant 141, 215-226.

Beaudoin, N., Serizet, C., Gosti, F. and Giraudat, J. (2000) Interactions between abscisic acid and ethylene signaling cascades. Plant Cell 12, 1103-1115.

Bemer, M., Karlova, R., Ballester, A.R., Tikunov, Y.M., Bovy, A.G., Wolters-Arts, M., Rossetto Pde, B., Angenent, G.C. and de Maagd, R.A. (2012) The tomato FRUITFULL homologs TDR4/FUL1 and MBP7/FUL2 regulate ethylene-independent aspects of fruit ripening. Plant Cell 24, 4437-4451.

Benjamini, B.Y.a.Y., D. (2001) THE CONTROL OF THE FALSE DISCOVERY RATE IN MULTIPLE TESTING UNDER DEPENDENCY. The Annals of Statistics 29, 1165-1188.

Bidonde, S., Ferrer, M.A., Zegzouti, H., Ramassamy, S., Latche, A., Pech, J.C., Hamilton, A.J., Grierson, D. and Bouzayen, M. (1998) Expression and characterization of three tomato 1-aminocyclopropane-1-carboxylate oxidase cDNAs in yeast. Eur J Biochem 253, 20-26.

Bohm, V., Puspitasari-Nienaber, N.L., Ferruzzi, M.G. and and Schwartz, S.J. (2002) Trolox Equivalent Antioxidant Capacity of Different Geometrical Isomers of a-Carotene, b-Carotene, Lycopene, and Zeaxanthin. J. Agric. Food Chem. 50, 221-226.

Brandi, F., Bar, E., Mourgues, F., Horvath, G., Turci, E., Giuliano, G., Liverani, A., Tartarini, S., Lewinsohn, E. and Rosati, C. (2011) Study of 'Redhaven' peach and its white-fleshed mutant suggests a key role of CCD4 carotenoid dioxygenase in carotenoid and norisoprenoid volatile metabolism. BMC plant biology 11, 24.

Brummell, D.A., Harpster, M.H., Civello, P.M., Palys, J.M., Bennett, A.B. and Dunsmuir, P. (1999) Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. Plant Cell 11, 2203-2216.
Brummell, D.A.a.H., M.H. (2001) Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol Biol.* 47, 311-340.

Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J.M., Bennett, A.B. and Powell, A.L. (2009) Ripening-regulated susceptibility of tomato fruit to Botrytis cinerea requires NOR but not RIN or ethylene. *Plant Physiol* 150, 1434-1449.

Carbone, F., Pizzichini, D., Giuliano, G., Rosati, C. and Perrotta, G. (2005) Comparative profiling of tomato fruits and leaves evidences a complex modulation of global transcript profiles. *Plant Sci* 169, 165-175.

Carrari, F., Baxter, C., Usadel, B., Urbanczyk-Wochniak, E., Zanor, M.I., Nunes-Nesi, A., Nikiforova, V., Centro, D., Ratzka, A., Pauly, M., Sweetlove, L.J. and Fernie, A.R. (2006) Integrated analysis of metabolite and transcript levels reveals the metabolic shifts that underlie tomato fruit development and highlight regulatory aspects of metabolic network behavior. *Plant Physiol* 142, 1380-1396.

Chung, M.Y., Vrebalov, J., Alba, R., Lee, J., McQuinn, R., Chung, J.D., Klein, P. and Giovannoni, J. (2010) A tomato (Solanum lycopersicum) APETALA2/ERF gene, SlAP2a, is a negative regulator of fruit ripening. *Plant J* 64, 936-947.

Cline, M.S., Smoot, M., Cerami, E., Kuchinsky, A., Landys, N., Workman, C., Christmas, R., Avila-Campilo, I., Creech, M., Gross, B., Hanspers, K., Isserlin, R., Kelley, R., Killcoyne, S., Lotia, S., Maere, S., Morris, J., Ono, K., Pavlovic, V., Pico, A., Vailaya, A., Wang, P., Adler, A., Conklin, B.R., Hood, L., Kuiper, M., Sander, C., Schmulevich, I., Schwikowski, B., Warner, G.J., Ideker, T., and Bader, G.D. (2007) Integration of biological networks and gene expression data using Cytoscape. *Nature Protocol* 2, 2366-2382.

Cordes, S., Deikman, J., Margossian, L.J. and Fischer, R.L. (1989) Interaction of a developmentally regulated DNA-binding factor with sites flanking two different fruit-ripening genes from tomato. *Plant Cell* 1, 1025-1034.

Curvers, K., Seifi, H., Mouille, G., de Rycke, R., Asselbergh, B., Van Hecke, A., Vanderschaeghe, D., Hofte, H., Callewaert, N., Van Breusegem, F. and Hofte, M. (2010) Abscisic acid deficiency causes changes in cuticle permeability and pectin composition that influence tomato resistance to Botrytis cinerea. *Plant Physiol* 154, 847-860.

D'Esposito, D., Ferriello, F., Molin, A.D., Diretto, G., Sacco, A., Minio, A., Barone, A., Di Monaco, R., Cavella, S., Tardella, L., Giuliano, G., Delledonne, M., Fruscianti, L. and Ercolano, M.R. (2017) Unraveling the complexity of transcriptomic, metabolomic and quality environmental response of tomato fruit. *BMC Plant Biology* 17, 66.

Dellapenna, D., Kates, D.S. and Bennett, A.B. (1987) Polygalacturonase Gene Expression in Rutgers, rin, nor, and Nr Tomato Fruits. *Plant Physiol* 85, 502-507.

Diretto, G., Al-Babili, S., Tavazza, R., Scossa, F., Papacchioni, V., Migliore, M., Beyer, P. and Giuliano, G. (2010a) Transcriptional-metabolic networks in {beta}-carotene-enriched potato tubers: the long and winding road to the "Golden" phenotype. *Plant Physiol* 154, 899-912.

Diretto, G., Al-Babili, S., Tavazza, R., Scossa, F., Papacchioni, V., Migliore, M., Beyer, P. and Giuliano, G. (2010b) Transcriptional-metabolic networks in {beta}-carotene-enriched potato tubers: the long and winding road to the "Golden" phenotype. *Plant Physiol.*
Enfissi, E.M., Barneche, F., Ahmed, I., Lichtle, C., Gerrish, C., McQuinn, R.P., Giovannoni, J.J., Lopez-Juez, E., Bowler, C., Bramley, P.M. and Fraser, P.D. (2010) Integrative transcript and metabolite analysis of nutritionally enhanced DE-ETIOLATED1 downregulated tomato fruit. Plant Cell 22, 1190-1215.

Fasano, C., Diretto, G., Aversano, R., D'Agostino, N., Di Matteo, A., Frusciante, L., Giuliano, G. and Carputo, D. (2016) Transcriptome and metabolome of synthetic Solanum autotetraploids reveal key genomic stress events following polyploidization. New Phytol 210, 1382-1394.

Fernandez-Moreno, J.P., Tzfadia, O., Forment, J., Presa, S., Rogachev, I., Meir, S., Orzaez, D., Aharoni, A. and Granell, A. (2016) Characterization of a New Pink-Fruited Tomato Mutant Results in the Identification of a Null Allele of the SIMYB12 Transcription Factor. Plant Physiol 171, 1821-1836.

Fridman, E., Carrari, F., Liu, Y.S., Fernie, A.R. and Zamir, D. (2004) Zooming in on a quantitative trait for tomato yield using interspecific introgressions. Science 305, 1786-1789.

Fu, D.Q., Zhu, B.Z., Zhu, H.L., Jiang, W.B. and Luo, Y.B. (2005) Virus-induced gene silencing in tomato fruit. Plant J 43, 299-308.

Fujisawa, M., Nakano, T., Shima, Y. and Ito, Y. (2013) A large-scale identification of direct targets of the tomato MADS Box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening. The Plant Cell 25, 371-386.

Galpaz, N., Wang, Q., Menda, N., Zamir, D. and Hirschberg, J. (2008) Abscisic acid deficiency in the tomato mutant high-pigment 3 leading to increased plastid number and higher fruit lycopene content. Plant J 53, 717-730.

Gascuel, Q., Diretto, G., Monforte, A.J., Fortes, A.M. and Granell, A. (2017) Use of Natural Diversity and Biotechnology to Increase the Quality and Nutritional Content of Tomato and Grape. Front Plant Sci 8, 652.

Giliberto, L., Perrotta, G., Pallara, P., Weller, J.L., Fraser, P.D., Bramley, P.M., Fiore, A., Tavazza, M. and Giuliano, G. (2005) Manipulation of the blue light photoreceptor cryptochrome 2 in tomato affects vegetative development, flowering time, and fruit antioxidant content. Plant Physiol 137, 199-208.

Giovannoni, J., DellaPenna, D., Bennett, A., and Fischer, R. (1989) Expression of a chimeric polygalacturonase gene in transgenic rin (ripening inhibitor)tomato fruit results in polyuronide degradation but not fruit softening. The Plant Cell 1, 53-63.

Giovannoni, J., Nguyen, C., Ampofo, B., Zhong, S. and Fei, Z. (2017) The Epigenome and Transcriptional Dynamics of Fruit Ripening. Annu Rev Plant Biol 68, 61-84.

Giovannoni, J., Tanksley, V., Vrebalov, J., and Noensie, E. (2004) NOR gene for use in manipulation of fruit quality and ethylene response. US Patent 6, 347.

Giuliano, G. (2014) Plant carotenoids: genomics meets multi-gene engineering. Curr Opin Plant Biol 19C, 111-117.

Giuliano, G. (2017) Provitamin A biofortification of crop plants: a gold rush with many miners. Curr Opin Biotechnol 44, 169-180.

Giuliano, G., Al-Babili, S. and von Lintig, J. (2003) Carotenoid oxygenases: cleave it or leave it. Trends Plant Sci 8, 145-149.
Giuliano, G., Bartley, G.E. and Scolnik, P.A. (1993) Regulation of carotenoid biosynthesis during tomato development. *Plant Cell* **5**, 379-387.

Gupta, R., Min, C.W., Kramer, K., Agrawal, G.K., Rakwal, R., Park, K.H., Wang, Y., Finkemeier, I. and Kim, S.T. (2018) A Multi-Omics Analysis of Glycine max Leaves Reveals Alteration in Flavonoid and Isoflavonoid Metabolism Upon Ethylene and Abscisic Acid Treatment. *Proteomics* **18**, e1700366.

Han, S.Y., Kitahata, N., Sekimata, K., Saito, T., Kobayashi, M., Nakashima, K., Yamaguchi-Shinozaki, K., Shinozaki, K., Yoshida, S. and Asami, T. (2004) A novel inhibitor of 9-cis-epoxycarotenoid dioxygenase in abscisic acid biosynthesis in higher plants. *Plant Physiol* **135**, 1574-1582.

Huisman, M.M.H., Schols, H.A. and Voragen, A.G.J. (1998) Cell wall polysaccharides from soybean (Glycine max.) meal. Isolation and characterisation. *Carbohydrate polymers* **37**, 87-95.

Iijima, Y., Nakamura, Y., Ogata, Y., Tanaka, K., Sakurai, N., Suda, K., Suzuki, T., Suzuki, H., Okazaki, K., Kitayama, M., Kanaya, S., Aoki, K. and Shibata, D. (2008) Metabolite annotations based on the integration of mass spectral information. *Plant J* **54**, 949-962.

Itkin, M., Seybold, H., Breitel, D., Rogachev, I., Meir, S. and Aharoni, A. (2009) TOMATO AGAMOUS-LIKE 1 is a component of the fruit ripening regulatory network. *Plant J* **60**, 1081-1095.

Ito, Y., Nishizawa-Yokoi, A., Endo, M., Mikami, M., Shima, Y., Nakamura, N., Kotake-Nara, E., Kawasaki, S. and Toki, S. (2017) Re-evaluation of the rin mutation and the role of RIN in the induction of tomato ripening. *Nat Plants* **3**, 866-874.

Ji, K., Kai, W., Zhao, B., Sun, Y., Yuan, B., Dai, S., Li, Q., Chen, P., Wang, Y., Pei, Y., Wang, H., Guo, Y. and Leng, P. (2014) SINCE1 and SICYP707A2: key genes involved in ABA metabolism during tomato fruit ripening. *Journal of experimental botany* **65**, 5243-5255.

Jiang, Y. and Joyce, D.C. (2003) ABA effects on ethylene production, PAL activity, anthocyanin and phenolic contents of strawberry fruit. *Plant Growth Regulation* **39**, 171–174.

Joung, J.G., Corbett, A., Fellman, S., Tieman, D., Klee, H., Giovannoni, J.J. and Fei, Z. (2009) Plant MetGenMAP: an integrative analysis system for plant systems biology. *Plant Physiol* **151**, 1758-1768.

Karlova, R., Rosin, F., Busscher-Lange, J., Parapunova, V., Do, P., Fernie, A., Fraser, P., Baxter, C., Angenent, G.C. and de Maagd, R. (2011) Transcriptome and metabolite profiling show that APETALA2a is a major regulator of tomato fruit ripening. *Plant Cell* **23**, 923-941.

Klee, H.J. and Giovannoni, J.J. (2011) Genetics and control of tomato fruit ripening and quality attributes. *Annu Rev Genet* **45**, 41-59.

Kopka, J., Schauer, N., Krueger, S., Birkemeyer, C., Usadel, B., Bergmüller, E., Dormann, P., Weckwerth, W., Gibon, Y., Stitt, M., Willmitzer, L., Fernie, A.R. and Steinhauser, D. (2005) GMD@CSB.DB: the Golm Metabolome Database. *Bioinformatics* **21**, 1635-1638.

Kumar, R., Tamboli, V., Sharma, R. and Sreelakshmi, Y. (2018) NAC-NOR mutations in tomato Penjar accessions attenuate multiple metabolic processes and prolong the fruit shelf life. *Food Chem* **259**, 234-244.

This article is protected by copyright. All rights reserved
Li, S., Xu, H., Ju, Z., Cao, D., Zhu, H., Fu, D., Grierson, D., Qin, G., Luo, Y. and Zhu, B. (2017) The RIN-MC fusion of MADS-box transcription factors has transcriptional activity. *Plant Physiol.*

Lin, Z.F., Hong, Y.G., Yin, M.G., Li, C.Y., Zhang, K. and Grierson, D. (2008) A tomato HD-Zip homeobox protein, LeHB-1, plays an important role in floral organogenesis and ripening. *Plant Journal* **55**, 301-310.

Lionetti, V., Francocci, F., Ferrari, S., Volpi, C., Bellincampi, D., Galletti, R., D'Ovidio, R., De Lorenzo, G. and Cervone, F. (2010) Engineering the cell wall by reducing de-methyl-esterified homogalacturonan improves saccharification of plant tissues for bioconversion. *Proc Natl Acad Sci U S A* **107**, 616-621.

Lisec, J., Schauer, N., Kopka, J., Willmitzer, L. and Fernie, A.R. (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nat Protoc* **1**, 387-396.

Liu, M., Diretto, G., Pirrello, J., Roustan, J.P., Li, Z., Giuliano, G., Regad, F. and Bouzayen, M. (2014) The chimeric repressor version of an Ethylene Response Factor (ERF) family member, Sl-ERF.B3, shows contrasting effects on tomato fruit ripening. *New Phytol* **203**, 206-218.

Lohse, M., Bolger, A.M., Nagel, A., Fernie, A.R., Lunn, J.E., Stitt, M. and Usadel, B. (2012) RobiNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. *Nucleic Acids Research* **40**, W622-W627.

Lu, Y., Chen, Q., Bu, Y., Luo, R., Hao, S., Zhang, J., Tian, J. and Yao, Y. (2017) Flavonoid Accumulation Plays an Important Role in the Rust Resistance of Malus Plant Leaves. *Front Plant Sci* **8**, 1286.

Manning, K., Tor, M., Poole, M., Hong, Y., Thompson, A.J., King, G.J., Giovannoni, J.J. and Seymour, G.B. (2006) A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nat Genet* **38**, 948-952.

Martel, C., Vrebalov, J., Tafelmeyer, P. and Giovannoni, J.J. (2011) The Tomato MADS-Box Transcription Factor RIPENING INHIBITOR Interacts with Promoters Involved in Numerous Ripening Processes in a COLORLESS NONRIpening-Dependent Manner. *Plant Physiol* **157**, 1568-1579.

Martin, L.B.B., Romero, P., Fich, E.A., Domozych, D.S. and Rose, J.K.C. (2017) Cuticle Biosynthesis in Tomato Leaves Is Developmentally Regulated by Abscisic Acid. *Plant physiology* **174**, 1384-1398.

McGlasson, W.B. and Adato, I. (1976) Changes in the Concentrations of Abscisic Acid in Fruits of Normal and <i>Nr</i>, rin<i>&lt;/i&gt;</i> and <i>Nor</i>&lt;i&gt;Mutant Tomatoes During Growth, Maturation and Senescence. *Functional Plant Biology* **3**, 809-817.

Meng, C., Yang, D., Ma, X., Zhao, W., Liang, X., Ma, N. and Meng, Q. (2016) Suppression of tomato SINAC1 transcription factor delays fruit ripening. *Journal of plant physiology* **193**, 88-96.

Mizrahi, Y., Dostal, H.C., McGlasson, W.B. and Cherry, J.H. (1975) Effects of Abscisic Acid and Benzyladenine on Fruits of Normal and rin Mutant Tomatoes. *Plant Physiol* **56**, 544-546.

Moco, S., Bino, R.J., Vorst, O., Verhoeven, H.A., de Groot, J., van Beek, T.A., Vervoort, J. and de Vos, C.H. (2006) A liquid chromatography-mass spectrometry-based metabolome database for tomato. *Plant Physiol* **141**, 1205-1218.
Mori, S., Bernardi, R., Laurent, A., Resnati, M., Crippa, A., Gabrieli, A., Keough, R., Gonda, T.J. and Blasi, F. (2012) Myb-binding protein 1A (MYBBP1A) is essential for early embryonic development, controls cell cycle and mitosis, and acts as a tumor suppressor. *PloS one* 7, e39723.

Mou, W., Li, D., Bu, J., Jiang, Y., Khan, Z.U., Luo, Z., Mao, L. and Ying, T. (2016) Comprehensive Analysis of ABA Effects on Ethylene Biosynthesis and Signaling during Tomato Fruit Ripening. *PloS one* 11, e0154072.

Mou, W., Li, D., Luo, Z., Li, L., Mao, L. and Ying, T. (2018) SIAREB1 transcriptional activation of NOR is involved in abscisic acid-modulated ethylene biosynthesis during tomato fruit ripening. *Plant Sci* 276, 239-249.

Mou, W., Li, D., Luo, Z., Mao, L. and Ying, T. (2015) Transcriptomic Analysis Reveals Possible Influences of ABA on Secondary Metabolism of Pigments, Flavonoids and Antioxidants in Tomato Fruit during Ripening. *PloS one* 10, e0129598.

Nakashima, K., Takasaki, H., Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2012) NAC transcription factors in plant abiotic stress responses. *Biochim Biophys Acta* 1819, 97-103.

Nitsch, L., Kohlen, W., Oplaat, C., Charnikhova, T., Cristescu, S., Michieli, P., Wolters-Arts, M., Bouwmeester, H., Mariani, C., Vriezen, W.H. and Rieu, I. (2012) ABA-deficiency results in reduced plant and fruit size in tomato. *Journal of plant physiology* 169, 878-883.

Orfila, C., Huisman, M.M., Willats, W.G., van Alebeek, G.J., Schols, H.A., Seymour, G.B. and Knox, J.P. (2002) Altered cell wall disassembly during ripening of Cnr tomato fruit: implications for cell adhesion and fruit softening. *Planta* 215, 440-447.

Osorio, S., Alba, R., Damasceno, C.M., Lopez-Casado, G., Lohse, M., Zanor, M.I., Tohge, T., Usadel, B., Rose, J.K., Fei, Z., Giovannoni, J.J. and Fernie, A.R. (2011) Systems biology of tomato fruit development: combined transcript, protein, and metabolite analysis of tomato transcription factor (nor, rin) and ethylene receptor (Nr) mutants reveals novel regulatory interactions. *Plant Physiol* 157, 405-425.

Pecker, I., Gabbay, R., Cunningham, F.X., Jr. and Hirschberg, J. (1996) Cloning and characterization of the cDNA for lycopene beta-cyclase from tomato reveals decrease in its expression during fruit ripening. *Plant Mol Biol* 30, 807-819.

Pham, T.D., Bo, W., West, G., Lycett, G.W. and Tucker, G.A. (2007) Silencing of the major salt-dependent isoform of pectinesterase in tomato alters fruit softening. *Plant Physiol* 144, 1960-1967.

Qin, X. and Zeevaart, J.A. (1999) The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proc Natl Acad Sci U S A* 96, 15354-15361.

Rambla, J.L., Trapero-Mozos, A., Diretto, G., Rubio-Moraga, A., Granell, A., Gomez-Gomez, L. and Ahrazem, O. (2016) Gene-Metabolite Networks of Volatile Metabolism in Airen and Tempranillo Grape Cultivars Revealed a Distinct Mechanism of Aroma Bouquet Production. *Front Plant Sci* 7, 1619.

Ronen, G., Carmel-Goren, L., Zamir, D. and Hirschberg, J. (2000) An alternative pathway to beta-carotene formation in plant chromoplasts discovered by map-based cloning of beta and old-gold color mutations in tomato. *Proc Natl Acad Sci U S A* 97, 11102-11107.
Ronen, G., Cohen, M., Zamir, D. and Hirschberg, J. (1999) Regulation of carotenoid biosynthesis during tomato fruit development: expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant Delta. *Plant J* 17, 341-351.

Rosati, C., Aquilani, R., Dharmapuri, S., Pallara, P., Marusic, C., Tavazza, R., Bouvier, F., Camara, B. and Giuliano, G. (2000) Metabolic engineering of beta-carotene and lycopene content in tomato fruit. *Plant J* 24, 413-420.

Ross, A.R., Ambrose, S.J., Cutler, A.J., Feurtado, J.A., Kermode, A.R., Nelson, K., Zhou, R. and Abrams, S.R. (2004) Determination of endogenous and supplied deuterated abscisic acid in plant tissues by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry with multiple reaction monitoring. *Anal Biochem* 329, 324-333.

Saladie, M., Rose, J.K., Cosgrove, D.J. and Catala, C. (2006) Characterization of a new xyloglucan endotransglucosylase/hydrolase (XTH) from ripening tomato fruit and implications for the diverse modes of enzymic action. *Plant J* 47, 282-295.

Schauer, N., Semel, Y., Roessner, U., Gur, A., Balbo, I., Carrari, F., Pleban, T., Perez-Melis, A., Bruedigam, C., Kopka, J., Willmitzer, L., Zamir, D. and Fernie, A.R. (2006) Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. *Nat Biotechnol* 24, 447-454.

Schauer, N., Zamir, D. and Fernie, A.R. (2005) Metabolic profiling of leaves and fruit of wild species tomato: a survey of the Solanum lycopersicum complex. *Journal of experimental botany* 56, 297-307.

Seo, P.J., Lee, S.B., Suh, M.C., Park, M.J., Go, Y.S. and Park, C.M. (2011) The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in Arabidopsis. *Plant Cell* 23, 1138-1152.

Seymour, G.B., Ostergaard, L., Chapman, N.H., Knapp, S. and Martin, C. (2013) Fruit development and ripening. *Annu Rev Plant Biol* 64, 219-241.

Silletti, M.F., Petrozza, A., Stigliani, A.L., Giorio, G., Cellini, F., D’Ambrosio, C. and Carriero, F. (2013) An increase of lycopene content in tomato fruit is associated with a novel Cyc-B allele isolated through TILLING technology. *Molecular Breeding* 31, 665-674.

Soto, A., Ruiz, K.B., Ravaglia, D., Costa, G. and Torrigiani, P. (2013) ABA may promote or delay peach fruit ripening through modulation of ripening- and hormone-related gene expression depending on the developmental stage. *Plant Physiol Biochem* 64, 11-24.

Su, L., Diretto, G., Purgatto, E., Danoun, S., Zouine, M., Li, Z., Roustan, J.P., Bouzayen, M., Giuliano, G. and Chervin, C. (2015) Carotenoid accumulation during tomato fruit ripening is modulated by the auxin-ethylene balance. *BMC plant biology* 15, 114.

Sun, L., Yuan, B., Zhang, M., Wang, L., Cui, M., Wang, Q. and Leng, P. (2012) Fruit-specific RNAi-mediated suppression of SINCED1 increases both lycopene and beta-carotene contents in tomato fruit. *Journal of experimental botany* 63, 3097-3108.

Sun, L., Zhang, M., Ren, J., Qi, J., Zhang, G. and Leng, P. (2010) Reciprocity between abscisic acid and ethylene at the onset of berry ripening and after harvest. *BMC plant biology* 10, 257.
Sun, Y., Ji, K., Liang, B., Du, Y., Jiang, L., Wang, J., Kai, W., Zhang, Y., Zhai, X., Chen, P., Wang, H. and Leng, P. (2017) Suppressing ABA uridine diphosphate glucosyltransferase (SlUGT75C1) alters fruit ripening and the stress response in tomato. *Plant J* 91, 574-589.

Thimm, O., Blasing, O., Gibon, Y., Nagel, A., Meyer, S., Kruger, P., Selbig, J., Muller, L.A., Rhee, S.Y. and Stitt, M. (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant J* 37, 914-939.

Thompson, A.J., Jackson, A.C., Symonds, R.C., Mulholland, B.J., Dadswell, A.R., Blake, P.S., Burbidge, A. and Taylor, I.B. (2000) Ectopic expression of a tomato 9-cis-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. *Plant J* 23, 363-374.

Thompson, A.J., Tor, M., Barry, C.S., Vrebalov, J., Orfila, C., Jarvis, M.C., Giovannoni, J.J., Grierson, D. and Seymour, G.B. (1999) Molecular and genetic characterization of a novel pleiotropic tomato-ripening mutant. *Plant Physiol* 120, 383-390.

Tieman, D., Zhu, G., Resende Jr., M.R.F., Lin, T., Nguyen, C., Bies, D., Rambla, J.L., Ortiz Beltran, K.R., Taylor, M., Zhang, B., Ikeda, H., Liu, Z., Fisher, J., Zemach, I., Monfrote, A., Zamir, D., Granell, A., Kirst, M., Huang, S., and Klee, H. (2017) A chemical genetic roadmap to improved tomato flavor. *Science* 355, 391-394.

Tieman, D.M. and Handa, A.K. (1994) Reduction in Pectin Methylesterase Activity Modifies Tissue Integrity and Cation Levels in Ripening Tomato (Lycopersicon esculentum Mill.) Fruits. *Plant Physiol* 106, 429-436.

Tigchelaar, E.C., Tomes, M., Kerr, E.A., Barman, R.J. (1973) A new fruit ripening mutant, nonripening (nor). *Rep Tomato Genet Coop.* 23, 33-34.

Uluisik, S., Chapman, N.H., Smith, R., Poole, M., Adams, G., Gillis, R.B., Besong, T.M., Sheldon, J., Stiegelmeyer, S., Perez, L., Samsulrizal, N., Wang, D., Fisk, I.D., Yang, N., Baxter, C., Rickett, D., Fray, R., Blanco-Ulate, B., Powell, A.L., Harding, S.E., Craigon, J., Rose, J.K., Fich, E.A., Sun, L., Domozycz, D.S., Fraser, P.D., Tucker, G.A., Grierson, D. and Seymour, G.B. (2016) Genetic improvement of tomato by targeted control of fruit softening. *Nat Biotechnol* 34, 950-952.

Urbanczyk-Wochniak, E., Usadel, E., Thimm, O., Nunes-Nesi, A., Carrari, F., Davy, M., Bläsing, O., Kowalczyk, M., Weicht, D., Polinceusz, A., Meyer, S., Stitt, M. and Fernie, A.R. (2006) Conversion of MapMan to allow the analysis of transcript data from Solanaceous species: Effects of genetic and environmental alterations in energy metabolism in the leaf. *Plant Mol. Biol.* 60, 773-792.

Vrebalov, J., Pan, I.L., Arroyo, A.J., McQuinn, R., Chung, M., Poole, M., Rose, J., Seymour, G., Grandillo, S., Giovannoni, J. and Irish, V.F. (2009) Fleshy fruit expansion and ripening are regulated by the Tomato SHATTERPROOF gene TAGL1. *Plant Cell* 21, 3041-3062.

Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Schuch, W. and Giovannoni, J. (2002) A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. *Science* 296, 343-346.
Wakabayashi, K., Hoson, T. and Huber, D.J. (2003) Methyl de-esterification as a major factor regulating the extent of pectin depolymerization during fruit ripening: a comparison of the action of avocado (Persea americana) and tomato (Lycopersicon esculentum) polygalacturonases. *Journal of plant physiology* **160**, 667-673.

Wang, D., Samsulrizal, N.H., Yan, C., Allcock, N.S., Craighton, J., Blanco-Ulate, B., Ortega-Salazar, I., Marcus, S.E., Bagheri, H.M., Perez Fons, L., Fraser, P.D., Foster, T., Fray, R., Knox, J.P. and Seymour, G.B. (2019) Characterization of CRISPR Mutants Targeting Genes Modulating Pectin Degradation in Ripening Tomato. *Plant Physiol* **179**, 544-557.

Welsch, R., Wust, F., Bar, C., Al-Babili, S. and Beyer, P. (2008) A third phytoene synthase is devoted to abiotic stress-induced abscisic acid formation in rice and defines functional diversification of phytoene synthase genes. *Plant Physiol* **147**, 367-380.

Yeats, T.H., Buda, G.J., Wang, Z., Chehanovsky, N., Moyle, L.C., Jetter, R., Schaffer, A.A. and Rose, J.K. (2012) The fruit cuticles of wild tomato species exhibit architectural and chemical diversity, providing a new model for studying the evolution of cuticle function. *Plant J* **69**, 655-666.

Yu, H.N., Wang, L., Sun, B., Gao, S., Cheng, A.X. and Lou, H.X. (2015) Functional characterization of a chalcone synthase from the liverwort Plagiochasma appendiculatum. *Plant Cell Rep* **34**, 233-245.

Zanor, M.I., Osorio, S., Nunes-Nesi, A., Carrari, F., Lohse, M., Usadel, B., Kuhn, C., Bleiss, W., Giavalisco, P., Willmitzer, L., Sulpice, R., Zhou, Y.H. and Fernie, A.R. (2009) RNA interference of LIN5 in tomato confirms its role in controlling Brix content, uncovers the influence of sugars on the levels of fruit hormones, and demonstrates the importance of sucrose cleavage for normal fruit development and fertility. *Plant Physiol* **150**, 1204-1218.

Zhang, C., Wang, X., Zhang, F., Dong, L., Wu, J., Cheng, Q., Qi, D., Yan, X., Jiang, L., Fan, S., Li, N., Li, D., Xu, P. and Zhang, S. (2017) Phenylalanine ammonia-lyase2.1 contributes to the soybean response towards Phytophthora sojae infection. *Sci Rep* **7**, 7242.

Zhang, J.Y., Broeckling, C.D., Blancaflor, E.B., Sledge, M.K., Sumner, L.W. and Wang, Z.Y. (2005) Overexpression of WXP1, a putative Medicago truncatula AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (Medicago sativa). *Plant J* **42**, 689-707.

Zhang, M., Yuan, B. and Leng, P. (2009) The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *Journal of experimental botany*.

Zhang, Y., Butelli, E., De Stefano, R., Schoonebeek, H.J., Magusin, A., Pagliarani, C., Wellner, N., Hill, L., Orzaez, D., Granell, A., Jones, J.D. and Martin, C. (2013) Anthocyanins double the shelf life of tomatoes by delaying overripening and reducing susceptibility to gray mold. *Curr Biol* **23**, 1094-1100.

Zhang, Y.L.Q., Jiang L, Kai W, Liang B, Wang J, Du Y, Zhou X, Wang J, Zhang Y, Sun Y, Zhang L, Leng P (2018) Suppressing Type 2C Protein Phosphatases Alters Fruit Ripening and the Stress Response in Tomato. *Plant Cell Physiology* **1**, 142-154.
FIGURE LEGENDS

Figure 1: Carotenoid composition, shelf life and antioxidant activity of LCYb-overexpressing fruits.
A. Schematic representation of β-carotene/ABA biosynthesis.
B. Carotenoid content of WT and LCYb-overexpressing fruits at B+10. Minimum levels of detection were 0.01 mg/g DW. Data are the average ± stdev of 3 biological replicates.
C. Elongated shelf-life of LCYb-overexpressing fruits. Fruits were harvested at the B stage and kept at 20±2°C, 60±5% relative humidity for the indicated periods of time.
D. Antioxidant activity, measured at different ripening stages as Trolox equivalent antioxidant capacity (TEAC) in non-polar (left) and polar (right) extracts. MG: Mature Green; B: Breaker; B+4, +10, +15: days after Breaker stage. Data are the average ± stdev of 3 biological replicates. Asterisks indicate significant differences from WT according to a Student's t-test (*P < 0.05, **P < 0.01).

Figure 2: Altered firmness, water loss, cuticle thickness, cell wall and cuticle composition of LCYb-overexpressing fruits.
A. Flesh firmness at 5 ripening stages, measured with a hand-held penetrometer. Data are the average ± stdev of 10 biological replicates. B. Water loss between the MG and B+15 ripening stages. Data are the average ± stdev of 10 biological replicates. C. Amounts of the different cell wall fractions in WT and LCYb-overexpressing fruits at B+10. TCW: Total cell walls; WSS: Water soluble solids; CHASS: Chelating agent soluble solids; DASS: Dilute alkali soluble solids; CASS: concentrated alkali soluble solids. Data are the average ± stdev of at least 3 biological replicates. D. Methyl-esterified pectin content of cell walls at B+10. Identical amounts of carbohydrate were applied to nitrocellulose membranes and probed with the LM20 monoclonal antibody. E. Light microscopy of cuticles from WT and LCYb-overexpressing fruits at B+10. F. Cuticle thickness of WT and LCYb-overexpressing fruits at B+10. Data are the average ± stdev of 10 biological replicates. Significant differences from WT were evaluated using a Student's t-test (*P < 0.05, **P < 0.01).

Figure 3: ABA/ethylene metabolism and RIN/NOR expression during fruit ripening.

This article is protected by copyright. All rights reserved
A. ABA content of WT and LCYb-overexpressing fruits at different stages of ripening.
B. Ethylene production in WT and LCYb-overexpressing fruits at different stages of ripening.
C. ACO activity in extracts from WT and LCYb-overexpressing fruits at different stages of ripening.
D. RIN and NAC-NOR expression in WT and LCYb-overexpressing fruits, measured by qRT-PCR. Data are normalized on the expression level of the actin housekeeping gene.

Data are the average ± stdev of 3 (A, C, D, E) or 10 (B) biological replicates. Asterisks indicate statistical significance (*, 0.05 < P; **, 0.01 < P) in a Student’s t test.

**Figure 4: Mapman representation of transcriptional-metabolic perturbations in LCYb-overexpressing fruits at B+10.**

Genes are represented by squares, metabolites by circles. Red indicates induction and blue indicates repression in the LCYb1 and LCYb3 lines with respect to the WT. A: ABA metabolism/signal transduction (54 Genes, 10 metabolites); B: Ethylene metabolism/signal transduction (119 Genes, 1 metabolite). C: Cuticle biogenesis (11 Genes, 13 metabolites); D: Cell wall biogenesis (49 Genes, 1 metabolite).

**Figure 5: Network analysis of co-regulated traits in LCYb-overexpressing fruits at B+10.**

Correlation network of 1,566 traits differentially regulated in LCYb-overexpressing fruits at B+10 with respect to the corresponding WT stage. Specific nodes of interest, associated to fruit ripening, are highlighted, together with their node strengths (ns, Diretto et al, 2010). N=number of nodes. NS=Network strength (Diretto et al, 2010). Each node represents a transcript (circle), a metabolite (diamond) or a phenotypic trait (square). For more details, see “Materials and Methods”.

**Figure 6: Abamine treatment reverses the phenotype of LCYb-overexpressing fruits.**

A. ABA content in WT and LCYb-overexpressing fruits at B+10, mock-treated or treated with 1 mM abamine; B. Flesh firmness of WT and LCYb-overexpressing fruits at B+10, mock-treated or treated with 1 mM abamine; C. Ethylene emission of WT and LCYb-overexpressing fruits at B+10, mock-treated or treated with 1 mM abamine; D. Water loss of...
WT and LCYb-overexpressing fruits, mock-treated or treated with 1 mM abamine, between MG and B+10.
Data are the average ± stdev of 5 biological replicates. Asterisks indicate significant differences from WT according to Student's t-test (*P < 0.05, **P < 0.01).

Figure 7: Proposed model for the enhanced shelf-life phenotype in LCYb-overexpressing fruits.
Variables showing an increase in LCYb-overexpressing fruits are shown in red, those showing a decrease are shown in green.

SUPPLEMENTAL FIGURE LEGENDS
Supplemental Figure 1. AtLCYb expression in transgenic fruits.
Transgene expression was measured in fruits at 5 stages of ripening via Real Time qRT-PCR. For details see “Materials and Methods”.
MG: Mature Green; B: Breaker; B+4, +10, +15, +40: days after Breaker stage. Data are the average ± stdev of 3 biological replicates.
Supplemental Figure 2. Time elapsing between anthesis and the breaker stage of fruit maturation in WT and LCYb-overexpressors.
Data are the average ± stdev of at least 10 biological replicates.
Supplemental Figure 3: Mapman representations of transcriptional perturbations observed in LCYb-overexpressing fruits at the MG, B and B+10 stages.
Genes and metabolites are represented by squares and circles, respectively. Red indicates induction and green indicates repression in the LCYb1 and LCYb3 lines with respect to the WT. Data are represented as log2 of fold change.
Supplemental Figure 4. Venn diagram of up- and down- regulated genes in LCYb-overexpressing fruits at three stages of ripening.
MG: mature green; B: breaker; B+10: ten days post the breaker stage. Sub-lists of common genes within 2 or 3 stages of ripening are reported in Supplemental Table 12.
Supplemental Figure 5. Correlation network of 1,008 and 790 differentially regulated features using, respectively, ABA (A) and ethylene (B) as central hubs. Circles mark $\rho$ value boundaries. Nodes co-regulated with ABA and ethylene are shown. The list of all the nodes and their strengths is reported in Supplemental Tables 15-16 (A-B). Each node represents a transcript (circle), a metabolite (diamond) or a phenotypic trait (square); up- and dw-regulated nodes in transgenic vs WT fruits are shown in red and green, respectively; direct correlations are shown as red lines, while inverse correlations as blue lines. Lines joining nodes represent correlations >|0.90| and lengths are inversely proportional to the $\rho$ absolute values. Number of nodes (N) and network strength (NS) (Diretto at al, 2010) are shown. For more details, see “Materials and Methods”.

SUPPLEMENTAL TABLE LEGENDS
Supplemental Table 1: carotenoid composition in WT and LCYb-overexpressing fruits at B+10 by LC-DAD-MS.
The amount of carotenoid compounds are expressed in $\mu$g g-1 dry weight. LCYb1-2-3 are three independent OX lines. Data are the average ± std. dev. of 3 biological replicates. Asterisks indicate statistical significance (*, 0.05 < P; **, 0.01 < P) in a Student’s t test. nd: not detectable.

Supplemental Table 2. Cutin and wax composition ($\mu$g/cm2) of cuticles from WT and LCYb-overexpressing fruits at B+10.
Data are the average ± SD of at least three biological replicates (fruits). Asterisks mark values that differ significantly from WT in a Student's t-test (*P < 0.05, **P<0.01).

Supplemental Table 3. Relative amounts of phenylpropanoids measured in the cuticle and flesh of WT and LCYb-overexpressing fruits at B+10.
Data are the average ± SD of at least three biological replicates (fruits). Asterisks mark values that differ significantly from WT according a Student's t-test (*P < 0.05, **P<0.01).

Supplemental Table 4. Relative amounts of primary metabolites in WT and LCYb-overexpressing fruits at B+10.
Data are the average ± SD of at least three biological replicates (fruits). Asterisks mark values that differ significantly from WT according a Student's t-test (*P < 0.05, **P<0.01).

Supplemental Table 5: levels of ABA and ABA catabolites in WT and LCYb-overexpressing fruits at 5 ripening stages (MG, B, B+4, B+10 and B+15).

Values are expressed as fold internal standard and normalized on the WT level. Data are the average ± std. dev. of 3 biological replicates. Asterisks indicate statistical significance (*, 0.05 < P; **, 0.01 < P) in a Student’s t test. nd: not detectable.

Supplemental Table 6A: Up-regulated genes in LCYb-overexpressing MG fruits.

Supplemental Table 6B: Dw-regulated genes in LCYb-overexpressing MG fruits.

Supplemental Table 7A: Overrepresented GO terms in all three categories (P: biological process; F: molecular function; C: cellular component) of up-regulated genes in LCYb-overexpressing fruits at MG.

Supplemental Table 7B: Overrepresented GO terms in all three categories (P: biological process; F: molecular function; C: cellular component) of dw-regulated genes in LCYb-overexpressing fruits at MG.

Supplemental Table 8A: Up-regulated genes in LCYb-overexpressing B fruits.

Supplemental Table 8B: Dw-regulated genes in LCYb-overexpressing B fruits.

Supplemental Table 9A: Overrepresented GO terms in all three categories (P: biological process; F: molecular function; C: cellular component) of up-regulated genes in LCYb-overexpressing fruits at B.

Supplemental Table 9B: Overrepresented GO terms in all three categories (P: biological process; F: molecular function; C: cellular component) of dw-regulated genes in LCYb-overexpressing fruits at B.

Supplemental Table 10A: Up-regulated genes in LCYb-overexpressing B+10 fruits.

Supplemental Table 10B: DW-regulated genes in LCYb-overexpressing B+10 fruits.

Supplemental Table 11A: Overrepresented GO terms in all three categories (P: biological process; F: molecular function; C: cellular component) of up-regulated genes in LCYb-overexpressing fruits at B+10.
Supplemental Table 11B: Overrepresented GO terms in all three categories (P: biological process; F: molecular function; C: cellular component) of dw-regulated genes in LCYb-overexpressing fruits at B+10.

Supplemental Table 12: Common UP- and DW-regulated genes in LCYb-overexpressing fruits in MG/B, B/B+10, MG/B+10 or MG/B/B+10 stages of ripening.

Supplemental Table 13: Genes tested by quantitative Real Time PCR, and expression levels in WT and LCYb-overexpressing fruits at B+10 stage of ripening.

Data are the average ± std. dev. of 3 biological replicates. Asterisks indicate statistical significance (*, 0.05 < P; **, 0.01 < P) in a Student’s t test.

Supplemental Table 14. Correlation matrix of differentially represented genes/metabolites/phenotypes in LCYb-overexpressing B+10 fruits.

Supplemental Table 15. Co-regulation of ripening-associated genes with ABA and ethylene in LCYb-overexpressing fruits.

Supplemental Table 16. Pearson correlation coefficients (ρ ≥|0.90|) between differentially regulated genes/metabolites/phenotypes and ABA (A), or Ethylene (B) in LCYb-overexpressing B+10 fruits.

Supplemental Table 17: lycopene, β-carotene and ABA- molar concentrations in WT and LCYb-overexpressing B+10 tomato fruits.

Data are the average ± std. dev. of 3 biological replicates. Asterisks indicate statistical significance (*, 0.05 < P; **, 0.01 < P) in a Student’s t test. nd: not detectable.

Supplemental Table 18: ABA- and stress- response elements sites found in the promoters of ethylene-regulators.
Figure 1: Carotenoid composition, shelf life and antioxidant activity of LCYb-overexpressing fruits.
A. Schematic representation of β-carotene/ABA biosynthesis.
B. Carotenoid content of WT and LCYb-overexpressing fruits at B+10. Minimum levels of detection were 0.01 µg/g DW. Data are the average ± stdev of 3 biological replicates.
C. Elongated shelf-life of LCYb-overexpressing fruits. Fruits were harvested at the B stage and kept at 20±2°C, 60±5% relative humidity for the indicated periods of time.
D. Antioxidant activity, measured at different ripening stages as Trolox equivalent antioxidant capacity (TEAC) in non-polar (left) and polar (right) extracts. MG: Mature Green; B: Breaker; B+4, +10, +15: days after Breaker stage. Data are the average ± stdev of 3 biological replicates. Asterisks indicate significant differences from WT according to a Student's t-test (*P < 0.05, **P < 0.01).

This article is protected by copyright. All rights reserved.
Figure 2: Altered firmness, water loss, cuticle thickness, cell wall and cuticle composition of LCYb-overexpressing fruits.

A. Flesh firmness at 5 ripening stages, measured with a hand-held penetrometer. Data are the average ± stdev of 10 biological replicates.

B. Water loss between the MG and B+15 ripening stages. Data are the average ± stdev of 10 biological replicates.

C. Amounts of the different cell wall fractions in WT and LCYb-overexpressing fruits at B+10. TCW: Total cell walls; WSS: Water soluble solids; CHASS: Chelating agent soluble solids; DASS: Dilute alkali soluble solids; CASS: Concentrated alkali soluble solids; GalA: galacturonic acid. Data are the average ± stdev of at least 3 biological replicates.

D. Methyl-esterified pectin content of cell walls at B+10. Identical amounts of carbohydrate were applied to nitrocellulose membranes and probed with the LM20 monoclonal antibody.

E. Light microscopy of cuticles from WT and LCYb-overexpressing fruits at B+10.

F. Cuticle thickness of WT and LCYb-overexpressing fruits at B+10. Data are the average ± stdev of 10 biological replicates. Significant differences from WT were evaluated using a Student's t-test (*P < 0.05, **P < 0.01).
Figure 3: ABA/ethylene metabolism and RIN/NOR expression during fruit ripening.
A. ABA content of WT and LCYb-overexpressing fruits at different stages of ripening.
B. Ethylene production in WT and LCYb-overexpressing fruits at different stages of ripening.
C. ACO activity in extracts from WT and LCYb-overexpressing fruits at different stages of ripening.
D, E. RIN and NAC-NOR expression in WT and LCYb-overexpressing fruits, measured by qRT-PCR. Data are normalized on the expression level of the actin housekeeping gene.
Data are the average ± stdev of 3 (A, C, D, E) or 10 (B) biological replicates. Asterisks indicate statistical significance (*, 0.05 < P; **, 0.01 < P) in a Student’s t test.
Figure 4: Mapman representation of transcriptional-metabolic perturbations in LCYb-overexpressing fruits at B+10. Genes are represented by squares, metabolites by circles. Red indicates induction and blue indicates repression in the LCYb1 and LCYb3 lines with respect to the WT. A: ABA metabolism/signal transduction (54 Genes, 10 metabolites); B: Ethylene metabolism/signal transduction (119 Genes, 1 metabolite). C: Cuticle biogenesis (11 Genes, 13 metabolites); D: Cell wall biogenesis (49 Genes, 1 metabolite).
Figure 5: Network analysis of co-regulated traits in LCYb-overexpressing fruits at B+10.
Correlation network of 1,566 traits differentially regulated in LCYb-overexpressing fruits at B+10 with respect to the corresponding WT stage. Specific nodes of interest, associated to fruit ripening, are highlighted, together with their node strengths (ns, Diretto et al, 2010). N=number of nodes. NS=Network strength (Diretto et al, 2010). Each node represents a transcript (circle), a metabolite (triangle) or a phenotypic trait (square). For more details, see “Materials and Methods”. 
Figure 6: Abamine treatment reverses the phenotype of LCYb-overexpressing fruits.
A. ABA content in WT and LCYb-overexpressing fruits at B+10, mock-treated or treated with 1 mM abamine;
B. Flesh firmness of WT and LCYb-overexpressing fruits at B+10, mock-treated or treated with 1 mM abamine;
C. Ethylene emission of WT and LCYb-overexpressing fruits at B+10, mock-treated or treated with 1 mM abamine;
D. Water loss of WT and LCYb-overexpressing fruits, mock-treated or treated with 1 mM abamine, between MG and B+10.
Data are the average ± stdev of 5 biological replicates. Asterisks indicate significant differences from WT according to Student's t-test (*P < 0.05, **P < 0.01).
Figure 7: Proposed model for the enhanced shelf-life phenotype in LCYb-overexpressing fruits. Variables showing an increase in LCYb-overexpressing fruits are shown in red, those showing a decrease are shown in green.