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Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors

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Dietary consumption of anthocyanins, a class of pigments produced by higher plants, has been associated with protection against a broad range of human diseases. However, anthocyanin levels in the most commonly eaten fruits and vegetables may be inadequate to confer optimal benefits. When we expressed two transcription factors from snapdragon in tomato, the fruit of the plants accumulated anthocyanins at levels substantially higher than previously reported for efforts to engineer anthocyanin accumulation in tomato and at concentrations comparable to the anthocyanin levels found in blackberries and blueberries. Expression of the two transgenes enhanced the hydrophilic antioxidant capacity of tomato fruit threefold and resulted in fruit with intense purple coloration in both peel and flesh. In a pilot test, cancer-susceptible *Trp53*$^{-/-}$ mice fed a diet supplemented with the high-anthocyanin tomatoes showed a significant extension of life span.

Anthocyanins are naturally occurring polyphenols present in many foods that are commonly consumed as part of the human diet. They offer protection against certain cancers, cardiovascular disease and age-related degenerative diseases[1–4]. There is evidence that anthocyanins also have anti-inflammatory activity[5], promote visual acuity[6], and hinder obesity and diabetes[7].

The health-promoting effects of anthocyanins have most frequently been linked to their high antioxidant activities[8]. Nonetheless, recent reports suggest that some of the biological effects of anthocyanins and flavonols may be related to their ability to modulate mammalian cell signaling pathways[9,10]. Regardless of the mechanism, these beneficial effects depend on relatively high levels of dietary anthocyanins.

Because anthocyanins as well as other health-promoting phytonutrients are present in relatively large amounts in fresh fruit and vegetables, the National Cancer Institute of America initiated the five-a-day program more than 20 years ago. This promotes the consumption of at least five portions of fruit and vegetables per day. Disappointingly, data from the campaign websites (http://www.fruitsandveggiesmatter.gov/; http://apps.nccd.cdc.gov/5ADaySurveillance/) show that currently only ~23% of the US population reach this level of consumption, and the numbers of people consuming the recommended five daily portions of fruit and vegetables have declined over the last 10 years. These figures argue strongly for the development of strategies to increase the levels of health-promoting bioactive compounds such as anthocyanins in the fruits and vegetables that people consume in substantial amounts.

Tomato is an excellent candidate for transgenic enhancement of flavonoid content. It is an important food crop worldwide and its levels of flavonoids (which include anthocyanins) are considered suboptimal, with only small amounts of naringenin chalcone and rutin accumulating in tomato peel[11]. Flavonoids represent an important source of hydrophilic dietary antioxidants, whereas the most abundant antioxidant in tomato fruit is lycopene, a lipophilic antioxidant. Generally, foods rich in both soluble and membrane-associated antioxidants are considered to offer the best protection against disease[12].

Although most tomato cultivars do not produce anthocyanins in fruit, low, light-dependent accumulation of anthocyanins in sectors of the skin has been achieved by introgression of a trait from a wild relative of tomato[13]. Genetic engineering strategies have also provided modest success; silencing of the tomato *DET1* gene, which represses light-mediated development, increases flavonoid levels up to 3.5-fold[14]. Constitutive, high-level activity of chalcone isomerase in tomato resulted in up to 78-fold increases in the levels of flavonols in fruit peel[11]. However, because peel accounts for only about 5% of fruit mass, the total levels were no more than 300 μg per g fresh weight.

Generally, transcription factors that regulate the expression of the genes involved in entire metabolic pathways provide effective tools for engineering high levels of metabolites[15,16]. Consequently, overexpression of the *ANT1* gene encoding a transcription factor that regulates anthocyanin production in tomato resulted in limited purple coloring on the skin and the cell layers immediately beneath the skin[17]. Overexpression of *Lc* and *C1*, two regulatory genes that encode transcription factors that control anthocyanin biosynthesis in maize, resulted in tomato fruit containing increased levels of flavonols (130 μg per g fresh weight), but unexpectedly, no anthocyanin accumulation was noted[18]. Although regulatory proteins offer the greatest potential to enhance anthocyanin biosynthesis, their

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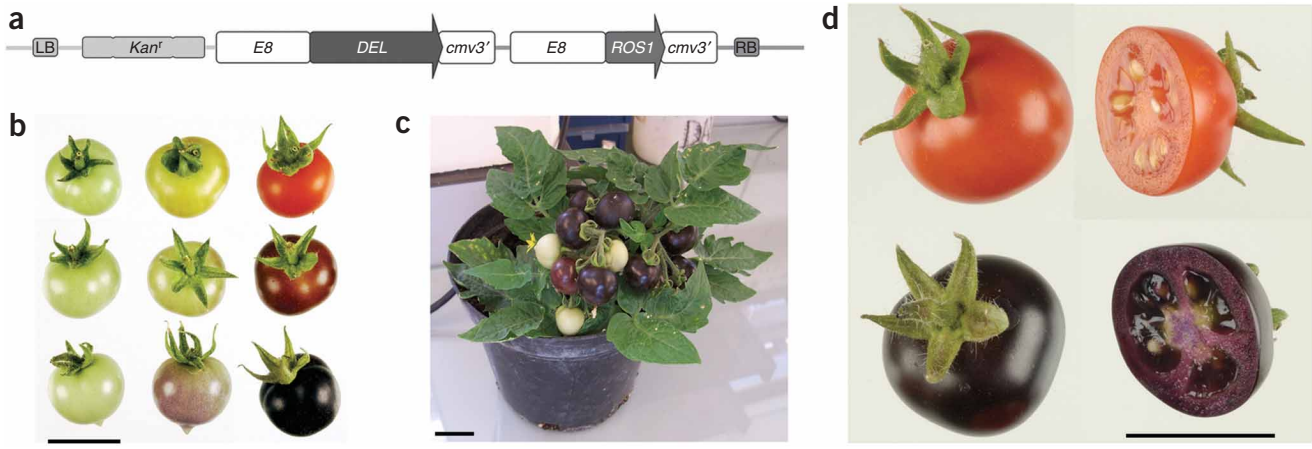


Figure 1 Fruit-specific phenotypes of T1 generation tomatoes (cv. MicroTom) expressing both *Del* and *Ros1* under the control of the *E8* promoter. (**a**) Map of T-DNA region of the binary vector used for transformation. LB, left T-DNA border region; RB, right T-DNA border region; Kan^r, *nptII* gene conferring kanamycin resistance under the control of the *nos* promoter; cmv3′, terminator region of cauliflower mosaic virus. (**b**) Phenotypic analysis of wild-type (upper row), *Del/Ros1*C (middle) and *Del/Ros1*N (lower) tomato fruit harvested at the green (left column), breaker (middle) and red (right) ripening stages. (**c**) *Del/Ros1*N tomato plant showing fruit at different stages of ripening. (**d**) Whole and cross-section of ripe wild-type and *Del/Ros1*N tomato fruit. All scale bars, 2 cm.

specificity may differ in different plant species. Differences in their efficacy in inducing anthocyanin production and in their target gene specificity have been documented[19–22], and explain the lack of anthocyanin production after expression of *Lc* and *C1* in tomato fruit[18].

We set out to produce tomato fruit with substantially elevated levels of anthocyanins by harnessing the appropriate target specificity of selected transcription factors. To do this, we expressed the Delila (*Del*) and Rosea1 (*Ros1*) genes from the snapdragon *Antirrhinum majus* in the fruit of transgenic tomatoes. *Del* encodes a basic helix-loop-helix transcription factor and *Ros1* encodes a MYB-related transcription factor[20,23] that interact to induce anthocyanin biosynthesis in snapdragon flowers.

We prepared a binary vector containing the *Del* and *Ros1* cDNAs each under the control of the fruit-specific *E8* promoter (**Fig. 1a**). Tomato (*Solanum lycopersicum* cv. MicroTom) leaf discs were transformed with the construct and four primary transformants, designated *Del/Ros1* lines C, N, Y and Z, were investigated in detail. The presence of the transgenes was confirmed by both DNA gel blots and PCR analysis of genomic DNA. The transgenes were inherited stably in later generations and could be transferred to other genetic backgrounds with no loss of phenotype (Money Maker, Ailsa Craig and VF36) by cross-pollination (**Supplementary Fig. 1** online). The high-anthocyanin, purple phenotype has been introduced into the Money Maker background and has been maintained through five generations in this commercial genetic background (Diego Orzaez and C.M., unpublished results).

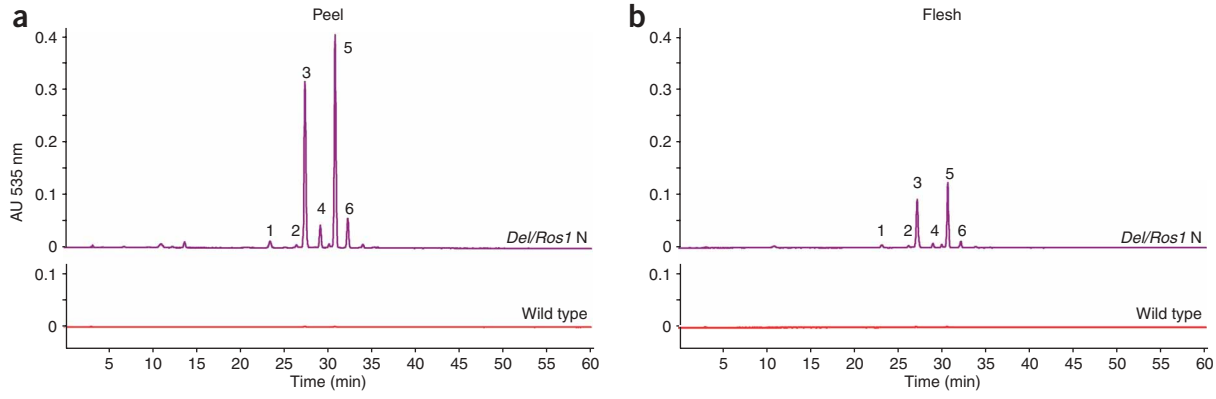
Del/Ros1 primary transformants developed normally during vegetative growth and were indistinguishable from controls. Stems and leaves showed no abnormal anthocyanin accumulation. Transgenic fruit developed normally and started to show visible signs of purple pigmentation only at the end of the mature green stage (**Fig. 1b**). Pigmentation matched the expression pattern of the *E8* gene[24] and intensified rapidly in the few days after its onset, initially associated with the vascular tissue but quickly extending to peel, pericarp and inner flesh (**Fig. 1c**,**d**). At maturity, the different transgenic tomato lines showed a range of phenotypes, with medium (*Del/Ros1*Z), strong (*Del/Ros1*C and *Del/Ros1*Y) or very strong accumulation of anthocyanin (*Del/Ros1*N). Fruit from individual plants of the T1 and T2 generations progressively showed more intense pigmentation than those from the T0 generation, as the transgenes were brought to homozygosity.

The total anthocyanin content was determined in the fruit of the hemizygous T0 *Del/Ros1* lines and compared to those in wild-type MicroTom fruit (**Supplementary Fig. 2a** online). The highest concentrations were found in the fruit of line N, which averaged 2.83 ± 0.46 mg of anthocyanin per g fresh weight. Anthocyanins were virtually undetectable in wild-type fruit. Ripe tomatoes from both transgenic *Del/Ros1*N and wild-type lines were harvested and peel and flesh were analyzed by high-performance liquid chromatography (HPLC) for their phenylpropanoid contents (**Fig. 2**). High levels of anthocyanins were detected in both peel and flesh of purple fruit (**Fig. 2a**,**b** and **Supplementary Fig. 2a**). The major anthocyanins were 3,5-diglucosides acylated with cinnamic acids (**Fig. 2c** and **Supplementary Fig. 3** online). Several of the methanol-soluble phenolic compounds we detected in the peel of purple fruit, including the main natural flavonol, rutin and other flavonol derivatives (**Fig. 2d**,**f**), were barely detectable in the peel of wild-type tomatoes (**Fig. 2d**). There were no increases in flavonols in the flesh of transgenic tomatoes (**Fig. 2e**). Comparative HPLC data showed that the same anthocyanins and flavonols accumulated in all four independent lines (**Supplementary Fig. 2b**,**c**).

The alterations in gene expression induced by *Del* and *Ros1* in fruit from the hemizygous line N were determined by comparison to control fruit by suppression subtractive hybridization, which identifies genes expressed in one tissue type but not in another. Differentially expressed genes in tomato fruit are listed in **Supplementary Table 1** online. Expression of *Del* and *Ros1* increased the transcript levels of almost all of the genes encoding enzymes required for anthocyanin biosynthesis, genes encoding enzymes required for side-chain modification such as a putative anthocyanin acyltransferase and two genes likely involved in the transport of anthocyanins into the vacuole, including a putative anthocyanin transporter[17].

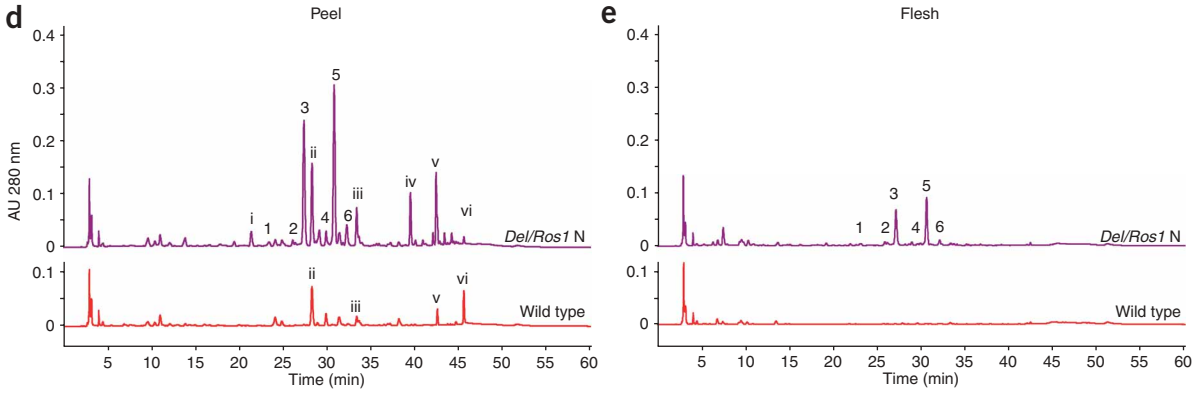
The differential expression of the genes identified by suppression subtractive hybridization was validated by RNA gel blot hybridization (**Fig. 3a**,**b**). Northern blot analysis also verified the expression of *Del* and *Ros1* and demonstrated the induction of expression of the gene

encoding flavanone 3-hydroxylase (*F3H*) by *Del* and *Ros1*, as this gene was not identified through the suppression subtractive hybridization protocol. With the exception of *PAL*, there was no detectable expression of any of these anthocyanin biosynthetic genes in wild-type tomato fruit. The induction of expression of these genes was confirmed by microarray analysis for all the independent transformed lines (**Supplementary Table 2** online). The induction of *PAL* expression by *Del* and *Ros1* significantly ($P < 0.01$) enhanced PAL enzyme activity, increasing it 200-fold in *Del/Ros1* line N (**Fig. 3c**). The transcription factors also increased chalcone isomerase activity threefold but only transiently during fruit development (**Supplementary Fig. 4** online).



c

	Peak identification	λ_{max} (nm)	$[M+H]^+$ (*m/z*)	Detected fragments
1	Delphinidin 3-(caffeoyl)-rutinoside-5-glucoside	529	935.24	773.23 (M+Glc), 465.11 (dpd+Glc), 303.05 (dpd)
2	Delphinidin 3-(cis-coumaroyl)-rutinoside-5-glucoside	533	919.27	757.22 (M+Glc), 465.12 (dpd+Glc), 303.06 (dpd)
3	Delphinidin 3-(trans-coumaroyl)-rutinoside-5-glucoside	529	919.28	757.19 (M+Glc), 465.11 (dpd+Glc), 303.05 (dpd)
4	Delphinidin 3-(feruloyl)-rutinoside-5-glucoside	529	949.24	787.21 (M+Glc), 465.10 (dpd+Glc), 303.06 (dpd)
5	Petunidin 3-(trans-coumaroyl)-rutinoside-5-glucoside	531	933.26	771.21 (M+Glc), 479.10 (ptd+Glc), 317.07 (ptd)
6	Petunidin 3-(feruloyl)-rutinoside-5-glucoside	530	963.27	801.23 (M+Glc), 479.13 (ptd+Glc), 317.07 (ptd)



f

	Peak identification	λ_{max} (nm)	$[M+H]^+$ (*m/z*)	Detected fragments
i	Myricetin-rhamnoglucoside	263/356	627.18	481.12 (M+Rha), 319.06 (M)
ii	Quercetin-rutinoside (rutin)	264/354	611.52	465.10 (Q+Glc), 303.04 (Q)
iii	Kaempferol-rhamnoglucoside	265/345	595.19	449.13 (K+Glc), 287.07 (K)
iv	Myricetin derivate	316	773.13	481.14 (M+Rha), 319.06 (M)
v	Quercetin derivate	315	757.23	465.12 (Q+Glc), 303.06 (Q)
vi	Naringenin chalcone	369	273.32	

Figure 2 Comparative analysis of phenylpropanoid content and composition. HPLC chromatogram of methanol extracts from *Del/Ros1*N (purple line) and wild-type (red line) tomato fruit. HPLC analysis, recorded at 535 nm (**a** and **b**) or 280 nm (**d** and **e**) of extracts from peel (**a** and **d**) or flesh (**b** and **e**) of ripe fruit. Peaks marked with numbers represent anthocyanins and peaks marked with roman numerals represent other flavonoids. Classification and identification of methanol soluble compounds was performed based on PDA absorbance and ESI-Q-TOF mass spectrometry (**c** and **f**). The purified compounds were analyzed by HPLC and ESI-MS/MS. Spectral characteristics, molecular ions and fragments obtained are tabulated. Identification was confirmed by hydrolysis and HPLC analysis of the respective acyl and sugar moieties. M, myricetin; Q, quercetin; K, kaempferol; Glc, glucose; Rha, rhamnose; dpd, delphinidin; ptd, petunidin.

Differences in total antioxidant activity between transgenic and wild-type tomato fruit were measured using the trolox equivalent antioxidant capacity (TEAC). The activity of the water-soluble fraction (containing anthocyanins) in *Del/Ros1*N fruit was threefold higher than in the control (**Fig. 3d**) and was not obtained at the expense of the liposoluble antioxidants (extracted with acetone). The increase in antioxidant activity in line C was less pronounced but still significantly higher than controls (**Fig. 3d**).

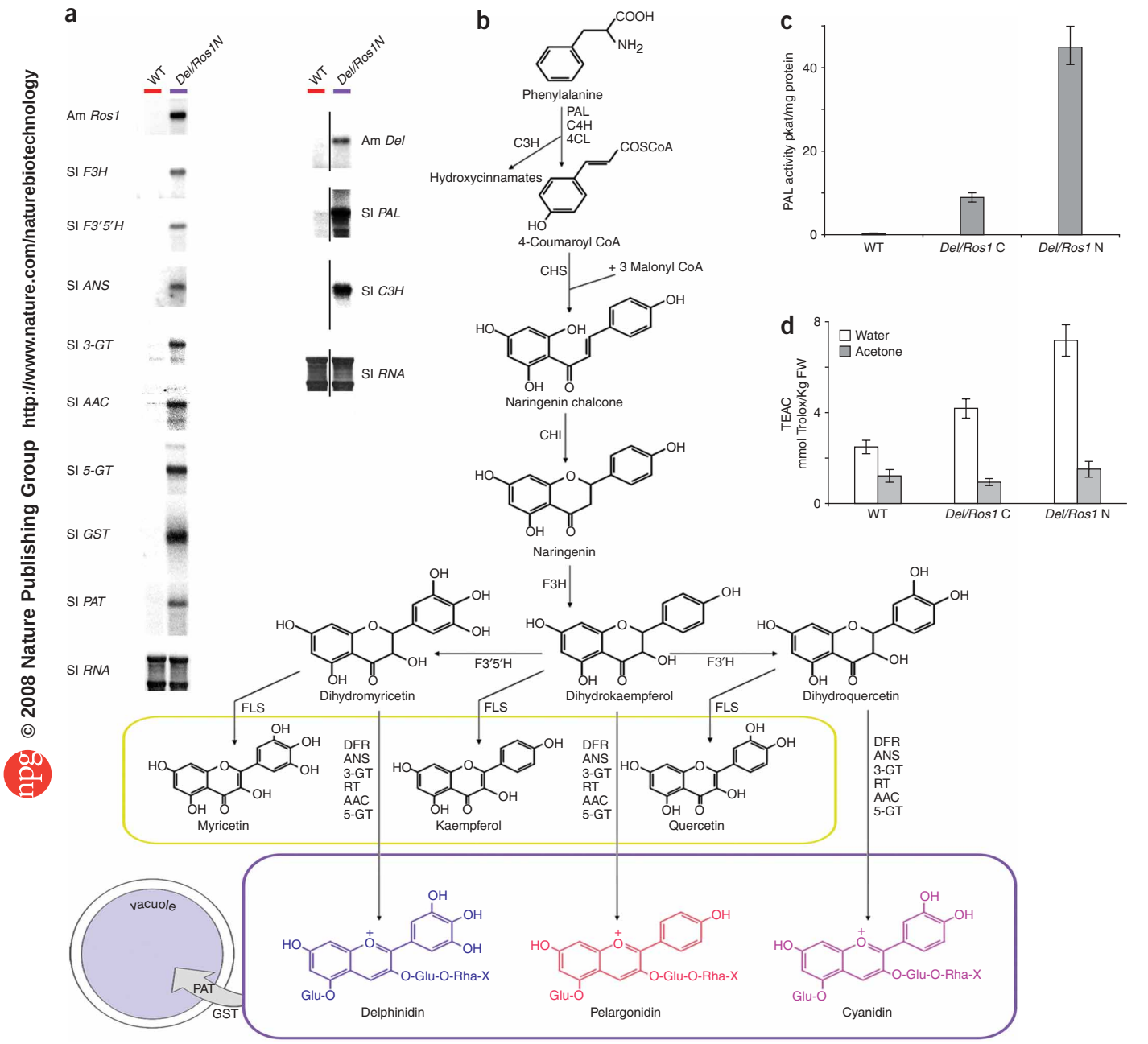


Figure 3 Expression of *Del* and *Ros1* causes the upregulation of genes required for anthocyanin biosynthesis and results in increased PAL activity and higher total antioxidant capacity. (**a**) Northern blots showing the differential expression of several anthocyanin biosynthetic genes identified by suppression subtractive hybridization. (**b**) Schematic representation of the anthocyanin biosynthetic pathway. Flavonoid classes relevant to this article are shown in boxes. Yellow box, flavonols; purple box, anthocyanins; PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate:coenzyme A ligase; C4H, cinnamate 4-hydroxylase; C3H, 4-coumarate 3-hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; F3′H, flavonoid-3′-hydroxylase; F3′5′H, flavonoid-3′5′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; 3-GT, flavonoid 3-O-glucosyltransferase; RT, flavonoid 3-O-glucoside-rhamnosyltransferase AAC, anthocyanin acyltransferase; 5-GT, flavonoid-5-glucosyltransferase; GST, glutathione S-transferase; PAT, putative anthocyanin transporter. PAT and GST may be involved in transport of anthocyanins into the vacuole (purple circle). X, acyl group on the 3-glucoside moiety of the anthocyanin. (**c**) Analysis of specific PAL activity in ripe tomato fruit from wild-type and transgenic lines *Del/Ros1*C and *Del/Ros1*N. Histograms represent mean values ± s.d. of separate measurements ($n = 3$). Results were confirmed in two additional independent experiments. (**d**) Analysis of hydrophilic and lipophilic antioxidant activity in ripe tomato fruit from wild-type and transgenic lines *Del/Ros1*C and *Del/Ros1*N. Data represent mean values ± s.d. and are derived from at least seven tomatoes per plant.

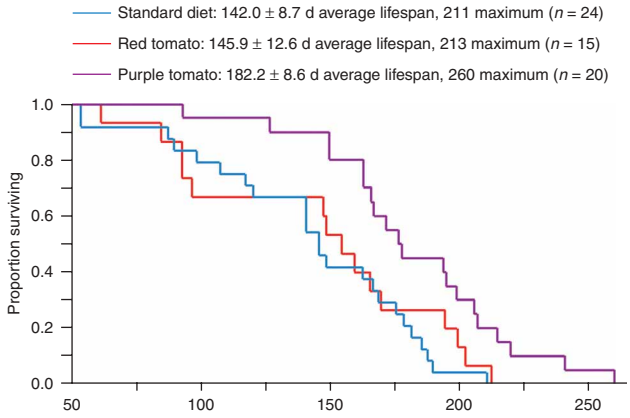


Figure 4 Life expectancy of *Trp53*$^{-/-}$ mice fed the standard diet or diets supplemented with 10% red or purple tomato powder. Kaplan-Meier survival plot; errors given in ± s.e.m. For statistical analysis of survival with respect to dietary treatment see **Supplementary Table 4**.

To investigate whether the levels of anthocyanins achieved were sufficient to offer health-promoting properties in a dietary context, we tested diets supplemented with tomato powder (10%) on mice, including a well-established mouse model for basic and applied cancer studies[25,26]. *Trp53*$^{-/-}$ knockout mice provide a useful tumorigenesis model because *p53* has been implicated in as many as half of all human cancers and mutant mice rapidly and reproducibly develop spontaneous tumors. We first fed 'wild-type' C57B16 mice with complete feed 'custom formula' for rodents, either without supplement or supplemented with 10% powder from freeze-dried red (control) tomatoes or purple (homozygous T2 *Del/Ros1*N) tomatoes. The content of carotenoids and flavonoids in the different pellet formulations were measured (**Supplementary Methods** and **Supplementary Table 3** online). Mice fed on the tomato-supplemented pellets (either red or purple) showed a slightly greater daily intake of food, compared to those fed on the standard diet. However, this compensated for the lower calorific value of the tomato-supplemented formulae, as the mice fed either red or purple tomato-supplemented pellets showed no additional weight gain compared to those on the standard pellets (**Supplementary Fig. 5** online).

Downregulation of *p53* expression or mutation of the *p53* locus has been found in a broad range of human cancers and cell proliferation disorders, indicating that p53 plays a key role in cell cycle regulation and tumor suppression. Indeed, mice lacking p53 spontaneously develop different types of cancer (mainly lymphomas) at an early age and are broadly used as a screening bioassay for potential cancer-protecting compounds[25,26]. p53 promotes antioxidant defense mechanisms, independently of its pro-oxidant activity (which is linked to the generation of ROS in the mitochondria of stressed cells). In nonstressed cells, low levels of p53 protect cells against oxidative damage, particularly to DNA, and promote survival and repair of more minor injuries. *Trp53*$^{-/-}$ knockout mice have an average life expectancy of about 140 d, although long-term dietary administration of strong antioxidants such as N-acetylcysteine (NAC) can significantly reduce the incidence of tumor induction and prolong their lifespan[27]. We compared the life expectancy of *Trp53*$^{-/-}$ knockout mice fed the standard diet, the diet supplemented with 10% red tomato powder or the diet supplemented with 10% purple tomato powder (**Fig. 4**). *Trp53*$^{-/-}$ knockout mice fed the standard diet had an average life span of 142 d and a maximum life span of 211 d (values very similar to those reported by others[25,26]). Animals fed on the pellets supplemented with red tomato powder showed no increase in their average life span (145.9 d). However, *Trp53*$^{-/-}$ knockout mice fed the pellets supplemented with the purple tomato powder had a significantly longer average life span (182.2 days; $P < 0.05$) and four mice lived for longer than 211 days, the maximum life span on this diet being 260 days. We are currently examining whether purified anthocyanins can offer similar lifespan extension or whether nutritional context has a substantial influence on the impact of dietary anthocyanins either through synergistic interactions with other constituents in food or effects on bioavailability.

We have shown that expression of the specific anthocyanin-regulating snapdragon transcription factors, Del and Ros1, induces the accumulation of high levels of anthocyanins in tomato. The reasons underlying the success of this regulatory combination are multifold. First, expression of both the MYB and the bHLH transcription factors gives rise to much higher levels of gene induction throughout all the tissues of the fruit compared to expression of either the MYB or the bHLH protein alone[17,28]. Second, Del and Ros1 activate a broader spectrum of genes in the phenylpropanoid/flavonoid pathway than Lc and C1 in tomato. *PAL* transcript levels and enzyme activity were increased substantially by Del/Ros1 in contrast to the effects of Lc/C1[18]. PAL activity determines flux through phenylpropanoid metabolism in Solanaceous species[29] and the effects of Del/Ros1 on PAL activity may explain the very high levels of anthocyanins that accumulated in *Del/Ros1* tomato fruit. Third, Del/Ros1 induced chalcone isomerase activity whereas Lc/C1 did not[18]. As chalcone isomerase activity may limit the flux through flavonoid metabolism in tomato skin[11], the ability of Del/Ros1 to induce the activity of this enzyme may also contribute to the high levels of anthocyanin or flavonol accumulation achieved. Fourthly, Del/Ros1 activated the gene encoding flavonoid 3′5′ hydroxylase (*F3′5′H*) whereas Lc/C1 did not, meaning that dihydrokaempferol rather than dihydromyricetin must be formed as an intermediate in flavonoid biosynthesis. In Solanaceous species, one biosynthetic enzyme, DFR, is specific for dihydromyricetin and will not accept dihydrokaempferol as a substrate, which may explain why Lc/C1 failed to induce anthocyanin accumulation in tomato fruit[18]. The differential specificity of transcription factors from different sources for their target genes, evidenced by the activity of the transcription factors controlling anthocyanin biosynthesis from different plant species, suggests that engineering strategies for phytonutrients should involve research into the specificity of proteins from different species to determine those most effective for achieving the metabolic objectives.

We have engineered the highest levels of anthocyanins yet reported in tomato fruit, and our preliminary animal study suggests that the levels of anthocyanins that accumulate in the tomatoes are sufficient to impart a substantial protective effect against cancer progression in *Trp53*$^{-/-}$ knockout mice, when included as part of their regular diet. The protective effects of dietary anthocyanins were not as great as those reported for NAC[27], although the tomato supplement was administered for a much shorter time (from weaning at ~14 d after birth), compared to NAC administration (from 2 weeks before mating of parents), and at a considerably lower dosage (anthocyanins were supplied at 80 mg [0.08 mmol] per kg per day, whereas NAC was supplied at 1g [6 mmol] per kg per day[27]). The beneficial effects of anthocyanins have, in the past, been attributed to their action as antioxidants, but from recent analysis, it seems unlikely that the protective effects of dietary flavonoids result from their direct activity as antioxidants[30]. However, anthocyanins may activate endogenous antioxidant defense systems and signaling pathways[9,10,31] and so act indirectly to delay oxidative damage and malignant progression.

Compared with their wild-type counterparts, our tomatoes provide highly characterized, isogenic material to evaluate the protective effects of dietary anthocyanins on other models of chronic disease, such as cardiovascular disease and the metabolic syndrome for which there are strong correlative data for protective effects of anthocyanins[32,33]. Clearly, our data also support the arguments for inclusion of foods containing high levels of anthocyanins in all long-term dietary regimens. To date, these have focused largely on consumption of berries such as blackcurrants, blackberries, raspberries, cranberries and blueberries[34]. The tomatoes we have engineered could contribute substantially to the hydrophilic antioxidant levels of human diets and might, as foods, be more widely adopted in preventive medicine strategies by healthy consumers than antioxidant supplements, such as NAC or vitamins, which are often viewed in the same way as conventional medicines.

METHODS

Experiments involving animals were performed in accordance with Italian Law (D.L.vo 116/92 and following additions), enforcing EU 86/609 Directive (Council Directive 86/609/EEC of 24 November 1986). The authority responsible for ensuring that the provisions of EU 86/609 Directive are properly carried out in Italy is the Ministry of Health. According to the regulatory requirements, the animal facility at IFOM-IEO was fully authorized by the Italian Ministry of Health (DM N° 65/2007-A – July 4, 2007) and a veterinarian, who is a specialist in laboratory animal science and medicine, was responsible for the well-being of the experimental animals.

Plasmid construction and plant transformation. A binary vector (pDEL.ROS) was constructed containing both the *Delila* and *Rosea1* cDNAs each under the control of the fruit-specific *E8* promoter from tomato using standard recombinant DNA techniques. The details of construction are provided in **Supplementary Methods**.

The binary plasmid pDEL.ROS was transferred to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating and used to transform tomato (var. MicroTom). Four independent transformed lines that showed accumulation of anthocyanin in fruit were generated and called *Del/Ros1* lines C,N,Y and Z. Southern blot and segregation analysis of the T1 generation of each line suggested that line N had 4 copies of the T-DNA inserted at two unlinked loci, line C had three copies of the T-DNA inserted at two unlinked loci, line Y had two copies of the T-DNA inserted at one locus and line Z had one copy of the T-DNA inserted at one locus.

Analysis and identification of anthocyanins and phenylpropanoids. Anthocyanins were extracted from chopped fruit with 5 ml of acidified (0.3% HCl, vol/vol) methanol for 24 h at 4 °C. After extraction, samples were centrifuged for 20 min at 5,000*g*. Anthocyanins in the supernatants were determined spectrophotometrically and expressed as mg of petunidin-3-(*p*-coumaroyl rutinoside)-5-glucoside (the main compound identified in *Del/Ros1* tomatoes) per g fresh weight, based on an extinction coefficient of 17,000 and a molecular weight of 934.

For HPLC analysis of phenylpropanoids, powdered plant material was extracted with 50% methanol and then with 100% methanol. Phenylpropanoids in the cleared extracts were analyzed by HPLC (Alliance, Waters) combined with a photodiode array detector (996, Waters). Separation was performed by reverse-phase chromatography on an Aqua C18, 5 µm, 4.6 × 250 mm column (Phenomenex) at 25 °C. The mobile phase was composed of 87% water, 3% acetonitrile (CAN) and 10% acetic acid (solvent A) or 40% water, 50% ACN and 10% acetic acid (solvent B) at a flow rate of 1 ml/min. The gradient was as follows: initial 6% B; 20 min, 20% B; 35 min, 40% B; 40 min, 60% B; 45 min, 90% B; and 60 min, 6% B. Absorbance spectra were recorded every 1 s, between 210 and 600 nm, with a bandwidth of 1.2 nm, and chromatograms were acquired at 280 and 535 nm. Data were analyzed using Waters Empower software.

For the identification of the major anthocyanins in *Del/Ros1*N tomato fruit, extracts were prepared from powdered tomato fruit with 50% methanol and then 100% methanol. Extracts were cleared by filtration through paper and then through a 0.22 µm membrane filter (Millipore). Extracts were concentrated to a final volume of 20 ml. A W600 pump system (Waters) with a preparative HPLC column (X-Bridge MS C18, 5 µm, 10 × 150 mm, Waters), maintained at 24 °C was used for separation. The mobile phase was composed of solvent A at a flow rate of 2 ml/min. For elution the following gradient was used: 95%A, 5% B × 80 min; 60%A, 40% B × 85–90 min; 100% B × 98 min; and 95%A, 5% B. Separated anthocyanins were analyzed by HPLC as described above. Purified substances were identified by electrospray ionization (ESI)-tandem mass spectrometry (MS/MS). Samples were injected by a syringe pump in 50% methanol with 1% formic acid. The nanoscale effluent from the syringe pump was directed to the NanoLockSpray source of a Q/ToF Premier hybrid orthogonal accelerated Time-of-Flight (oa-ToF) mass spectrometer (Waters, MS Technologies Centre). The MS operated in a positive ion mode at 80 °C and a cone gas flow of 30 liters/h. A voltage of ~2 kV was applied to the nano flow sample tip. The mass spectra were acquired with the ToF mass analyzer in V-mode of operation and spectra were integrated over 1 s intervals. MS and MS/MS data were acquired in a continuum mode using MassLynx 4.0 software (Waters, Technologies Centre). The instrument was calibrated with a multipoint calibration using selected fragment ions of the CID of Glu-Fibrinopeptide B (Sigma-Aldrich).

For the identification of acyl moieties, pure anthocyanin fractions were subjected to alkaline hydrolysis with 10% KOH for 30 min at 24 °C. The lysate was acidified to pH 1.0 with 2 M HCl and the decomposition products were extracted with ethyl acetate. The organic phase was evaporated to dryness and the phenylpropanoids were resuspended in solvent A for HPLC-analysis.

For identification of sugar moieties, pure anthocyanin fractions were subjected to acid hydrolysis with 2 M HCl for 30 min at 95 °C in a sealed vial. After cooling of the lysate in an ice bath the aglycones were extracted with 1-pentanol. The organic and the water phase were evaporated to dryness. The phenylpropanoids were resuspended in solvent A for HPLC analysis as described above. The sugar moieties were resuspended in water for HPLC-analysis.

Gene expression analysis. Total RNA samples to be used in suppression subtractive hybridization were obtained from fruit harvested at the turning, pink and red stages. To minimize interference from ripening-related genes, minor RNA amounts (11%) extracted from fruit at the breaker and overripe stages were also included in the wild-type, control RNA pool. PolyA+ RNA was purified from total RNA using mRNA purification kit (Amersham Biosciences) according to the manufacturer's instructions. Approximately 2 µg each of *Del/Ros1*N and wild-type polyA+ RNA were reverse transcribed into cDNA and further processed according to the protocol supplied with the Clontech PCR-Select cDNA Subtraction Kit. PCR products were cloned using T/A cloning vector (Invitrogen) and used to transform *Escherichia coli* cells (DH5α). Two hundred colonies were randomly selected for colony PCR amplification and the reaction products were spotted on four nylon membranes and probed in duplicate with radiolabeled cDNA obtained from either wild-type or *Del/Ros1*N transgenic MicroTom fruit. Clones showing changes in expression between the wild-type and transgenic samples were selected for DNA sequencing and validated by northern blot hybridization. Four 1.2% (wt/vol) agarose formaldehyde gels were prepared, each containing 15 µg of total RNA extracted from wild-type and *Del/Ros1*N tomato fruit. The RNA was transferred to nylon membranes and equal RNA loading and transfer were confirmed by methylene blue staining. The membranes were hybridized with labeled cDNA fragments corresponding to regulated transcripts isolated by suppression subtractive hybridization and obtained from *Eco*RI-digested plasmids. The radioactivity on the membranes was detected with a PhosphorImager (Fuji BAS 1000) or with Biomax film (Kodak). The membranes were stripped after each round of hybridization and exposed to a PhosphorImager to verify complete removal of the probe.

DNA microarrays. The PROFOOD oligonucleotide microarray was constructed using 1,034 70-mer oligonucleotides (Qiagen Operon), each representing a tomato expressed sequence tag (EST) selected by bioinformatic analysis. The 1,034 selected ESTs represented genes involved in metabolic and regulatory pathways related to fruit quality and nutritional value. Control oligonucleotides were included for background subtraction and normalization. Each

oligonucleotide was printed four times on amino-silane–coated glass slides (Corning) by a capillary spotting device (Cartesian Technologies). After printing, the slides were air-dried for several days, rehydrated and the DNA was cross-linked using an UV-cross linker at 150 m joules. The slides were soaked twice in 0.2% SDS for 2 min, twice in MQ water for 2 min and once in boiling MQ water for 2 min. After drying, the slides were rinsed three times in 0.2% SDS for 1 min and once in MQ water for 1 min. Finally, the slides were submerged in boiling MQ water for 2 s.

CY3- and CY5-labeled cDNA probes were prepared using 5 μg total RNA with a kit from Genisphere. Hybridizations were performed with CY3-labeled cDNA from duplicate biological samples of each individual *Del/Ros1* line and two control lines compared to a common CY5-labeled reference (that is, pooled cDNA composed of equal amounts from each individual line). All microarrays were prehybridized for 3 h at 45 °C before overnight hybridization in a 120 μl hybridization volume. All hybridization and washing steps were carried out using an automatic hybridization station (HybArray 12, Perkin Elmer). The slides were dried by centrifugation before scanning. Detection of the CY3 and CY5 signals was performed (ScanArray Express HT, Perkin Elmer). Spot identification and signal quantification were performed using Analytical Imaging Station AIS 4.0 software (IMAGING Research).

The background levels of signal in the microarray experiments were calculated from the raw data signals from oligonucleotide spots of nonplant origin. The mean values of these were subtracted from all others. Normalization was carried out against the median for each experiment. For statistical analysis of microarray data from the different tomato lines, mean values for genes from the biological replicates were used for pair-wise comparison between all values.

Total antioxidant activity. Homogenized tomato samples (1g) were each extracted twice with water. The pulp residue was reextracted twice in acetone. The TEAC, which is based on the ability of antioxidant molecules to quench the long-lived ABTS (2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonate, Sigma-Aldrich) radical cation, a blue-green chromophore with characteristic absorption at 734 nm, compared with that of trolox (6-hydroxy-2.5,7,8-tetramethylchroman-2-carboxylic acid, Fluka), a water-soluble vitamin E analog, was determined. Results were expressed as TEAC in mmol of trolox per kg of fresh weight.

Analysis of mouse models. Experimental subjects were WT and *Trp53*$^{-/-}$ mice of the C57/Bl6 strain[25]. The colony was bred in the animal facility of the Department of Experimental Oncology, IEO-IFOM, Milan. Animals were kept under standard conditions: air-conditioned room, 21 ± 1 °C, relative humidity 60 ± 10%, with a white-red light cycle (lights on from 08:30 to 20:30). Group housing was used to improve animals' welfare. Home cages were Plexiglas boxes (4 in 42 × 27 × 14 cm) with sawdust as bedding. Pellet food (Enriched Standard Diet purchased from Mucedola) and tap water were continuously available. Different batches of animals were used for each test.

Mice were fed with the different diets. Body weight and food consumption were measured twice a week. Life span of *Trp53*$^{-/-}$ mice was analyzed using JMP starter software (The Statistical Discovery Software), which considers the survival and reliability of experimental mice and is able to compare the longevity of the different treatment groups using a Chi square statistical test.

Freeze-dried tomato powder was provided to Mucedola to formulate the mouse pellets supplemented at 10% by weight of the standard formula with tomato powder by mixing the dried tomato powder with the dried diet, grinding and addition of water, wire drawing at 24 °C, drying at 32 °C for 18 h to reach 12% moisture content and vacuum packaging. The dried standard diet (4.8 kcal/g) formula included: wheat, soybean toast flour, corn flour, wheat straw, bent grass flour, fish flour, mineral mix, milk powder, soybean oil, D-L methionine, and vitamin mix.

Statistical methods used to assess *Trp53*$^{-/-}$ mouse survival. To assess the significance of the different survival times of *Trp53*$^{-/-}$ mice on the different diets, we used the Log-Rank Test of the χ^2 square values. The Log Rank Test places more weight on longer survival times and is most useful when the ratio of hazard functions in the groups being compared is approximately constant. P is the probability of being $> \chi^2$ and lists the probability of obtaining, by chance alone, a χ^2 value greater than the one computed if the survival functions are the same for all groups (**Supplementary Table 4** online).

Accession numbers. Array data were submitted to the Array Express MIAME database with the accession identifiers Array: PRI-PROFOOD = A-MEXP-1417 Experiment: PRI-PROFOOD = E-MEXP-1830.

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

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AUTHOR CONTRIBUTIONS

E.B. assembled the constructs for transformation, undertook the transformation experiments, performed all of the molecular biological analysis and contributed to the planning and writing of the paper. L.T. and M.G. performed the animal experiments with mice. H.-P.M., A.M. and S.P. performed the phenolic analysis and identification and contributed to writing the manuscript. E.G.W.M.S., R.D.H. and A.G.B. prepared the PROFOOD tomato microarray, undertook the comparative expression profiling and the comparative HPLC analysis in the different transgenic lines. J.L. performed HPLC analysis in the different transgenic lines. C.M. planned and designed the project and contributed to the writing of the manuscript.

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