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Metabolite profiling of carotenoid and phenolic pathways in mutant and transgenic lines of tomato: Identification of a high antioxidant fruit line

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Dedicated to Professor Rod Croteau on the occasion of his 60th birthday.

Abstract

Plant secondary metabolism is highly regulated within the major pathways to terpenoids, phenolics and alkaloids. Such regulation can occur at multiple levels from transcription through to the compartmentation of the product. However, the possibility exists for cross-talk between these pathways, the regulation of which is largely unknown at present. Such phenomena are important to understand in the application of plant breeding, where unintended effects of transgenesis or mutation can have an impact on the environment or human health. In an effort to improve dietary antioxidant content of crop plants, the tomato has been a major focus of effort for engineering both lipophilic antioxidants such as carotenoids and hydrophilic antioxidants such as flavonoid glycosides. In this study, a panel of transgenic and mutant tomato lines has been subjected to metabolite profiling in comparison with wild type Ailsa Craig for both carotenoids and phenolics. A range of mutants and transgenic lines were selected showing a range of phenotypes varying from down-regulation through to increased levels of lycopene and β-carotene. All mutants altered in structural genes for carotenoid biosynthesis showed that perturbations in carotenoid biosynthesis do not generally alter phenolic or flavonoids content significantly even when devoid of carotenoids. Reciprocally, the down-regulation of ferulate 5-hydroxylase had no effect on carotenoid content. In contrast mutants defective in light perception such as the high pigment (*hp*-1) and LA3771 possess elevated chlorogenic acid and rutin as well as increased carotenoid content. These lines can act as the hosts for further genetic manipulation for increased antioxidant content.

Keywords: *Lycopersicon esculentum*; Solanaceae; Tomato; Carotenoids; Flavonoids; Phenylpropanoids; Metabolite profiling

1. Introduction

The major groups of secondary metabolites, terpenoids, phenolics and alkaloids form the basis of many of the properties of plants. This huge diversity of biochemical pathways leads to the evolution of species and crop plants making specialist compounds that have an impact on the environment and nutrition and health. In recent years nutraceuticals and functional foods have attracted much attention, particularly with respect to protective dietary intake. The carotenoids and polyphenols and their bioactive products have been the main areas of focus, but others such as tocopherols, glucosinolates and ascorbate acid, for example have also attracted considerable attention.

Carotenoids, among the terpenoids, have accessory pigment functions in plants as well as roles as protectants. In human health it is mainly accepted that they have important dietary impact as lipophilic antioxidants. Since humans are

Abbreviations: d.p.b., days post breaker; PDA, photodiode array.

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unable to synthesise carotenoids de novo, we obtain them exclusively from the diet. Lycopene [**1**], the red pigment of tomato has received much attention. At least 85% of our dietary lycopene [**1**] comes from tomato fruit and tomato-based products, the remainder being obtained from watermelon, pink grapefruit, guava and papaya. Uptake of carotenoids from the diet has been studied for many years (reviewed by Rao and Agarwal, 1999). The bioavailability of dietary lycopene [**1**] appears to be dependent upon several factors. It is absorbed better from heat processed foods than unprocessed sources and also from lipid-rich diets (Bohm and Bitsch, 1999). Once ingested, lycopene [**1**] then appears in plasma, initially in the VLDL and chylomicron fractions and later in LDL and HDL. The highest levels are found in LDL. Serum concentrations, however, vary enormously, from 50 to 900 nM, with large interperson variations. Lycopene [**1**] is found in most human tissues, but is not deposited uniformly (Fraser and Bramley, 2004). These differences suggest that there are specific mechanisms for the preferential deposition of lycopene [**1**], particularly in the adrenals and testes. Several epidemiological studies have been published which show an inverse correlation between tomato-rich diets and the incidence of several cancers and coronary heart disease (Giovannucci et al., 1995; Sengupta and Das, 1999). The mode of action of lycopene [**1**] in alleviating CHD is thought to be due to its antioxidant properties, leading to the protection of serum lipoproteins to oxidation, although conclusive studies have yet to be reported (Rice-Evans et al., 1997a). β-Carotene [**2**] is a provitamin A carotenoid and its deficiency can cause xerophthalmia, blindness, and premature death (Mayne, 1996). As a result of their potential importance the carotenoids have long been targets for manipulation, Golden Rice being the most high profile example (Ye et al., 2000). Besides potential utility these lines have also given important insights into the regulation of carotenoid synthesis (Bramley, 2002). Although the control of gene expression is thought to be the main regulatory mechanism for these alterations in carotenoids, post-transcriptional regulation has also been reported, including feedback inhibition. The use of genetic manipulation of carotenogenesis in tomato has been used primarily for biotechnological reasons, but it has also facilitated investigations into these regulatory mechanisms, as well as into the effects of such perturbations on other isoprenoids such as gibberellins, tocopherols and sterols.

The phenolics are also potent antioxidants when tested in vitro (Rice-Evans et al., 1995, 1997b). Based on these findings, it was postulated that flavonoids may offer protection against major diseases such as coronary heart diseases and cancer (Hertog and Hollman, 1996). Attention has now focussed on their activity in vivo particularly the bioavailability of polyphenols through their uptake and excretion of their conjugated forms (Miyazawa et al., 1999; Rechner et al., 2002; van het Hof et al., 1999; de Vries et al., 1998). Furthermore, the relationship between biomarkers of the colonic biotransformation of ingested dietary polyphenols and the absorbed conjugated polyphenols, show that the majority of the in vivo forms derive from cleavage products of the action of colonic bacterial enzymes and subsequent metabolism in the liver. The spectrum of compounds observed, suggest that the cleavage products have a putative role as physiologically relevant bioactive components in vivo (Rechner et al., 2001).

Phenolics as antioxidants, in particular the flavonoids, have also been targets for enhancement in crops (Parr and Bolwell, 2000). Both structural genes and transcription factors have been manipulated, the latter because they play a prominent part in the overall regulation of the branches in the pathway (Vom Endt et al., 2002). A survey of regularly consumed fruit and vegetables showed that in contrast with those rich in anthocyanins, flavonones and flavonols, tomato which is rich in hydroxycinnamates, showed specific antioxidant activity in the lower range (Protoggente et al., 2002). With respect to flavonoids, tomato contains rutin (quercetin-3-rutinoside, **3**), kaempferol-3-*O*-rutinoside [**4**] and naringenin chalcone [**5**] (Crozier et al., 1997; Muir et al., 2001). Two strategies have proved successful in enhancing the level of these. In the first, petunia chalcone isomerase was expressed in tomato fruit resulting in enhanced levels of rutin at the expense of naringenin in the skin (Muir et al., 2001). This approach using a structural gene was complemented by an alternative strategy using heterologous expression of transcription factors LC and C1 from maize (Bovy et al., 2002). This approach resulted in accumulation of kaemferol and naringenin in the pericarp, a tissue from which they are usually absent. A further phenotype was the accumulation of anthocyanins in the leaves but not the fruit, which was attributed to an insufficient expression of the gene encoding flavanone-3′5′-hydroxylase in the fruit. Therefore there is potential for manipulating dietary phenolic antioxidants in crops making it imperative that the consequences on other pathways are established. The tomato pericarp also contains the hydroxycinnamates *p*-coumarate [**6**], caffeate [**7**], ferulate [**8**], sinapate [**9**] and chlorogenic acid [**10**]. Levels of these compounds have also been manipulated in programmes aimed at lowering monolignol intermediates and, as a consequence, lignin in cell walls (Anterola and Lewis, 2002).

Since the products of both pathways produce antioxidants, it is important to understand how manipulation of one may affect the other and the extent of any cross-talk between the two pathways. Therefore we have tested this hypothesis by metabolite profiling of transgenic and mutant lines of tomato altered in carotenoids. This was also prompted by our finding that the *rr* mutant of tomato is devoid of carotenoids, but still has a yellow colour due to 50% higher levels of naringenin compared with WT. In order to test the effect of manipulation of phenolic metabolism upon carotenoid content we produced a down-regulated line for CYP84, (ASF5H), respectively, since this is represented by a single gene in tomato. F5H was chosen so as to place a block in the pathway with potential elevation of ferulate [**8**], an important dietary antioxidant.

2. Results and discussion

2.1. Distribution of carotenoids and phenolics

Carotenoids are lipophilic pigments, found predominantly within the pericarp tissue of the tomato fruit. The skin tissue may contain low levels of carotenoids due to adhesion of pericarp cells during isolation. Phenylpropanoids such as chlorogenic [**10**] and caffeic [**7**] acids were detected in both pericarp and skin tissue. Flavonoids, however, were only detected in the skin tissue during the present study. Thus in order to determine the carotenoid contents of the tomato mutants a combination of skin and pericarp tissue were analysed simultaneously. In contrast, phenylpropanoid and flavonoid analyses were carried out on pericarp and skin tissue separately.

2.2. Levels of carotenoids and phenolics in mutant and transgenic lines

The carotenoid content of seven tomato lines (both mutant and transgenic; Table 1) with altered carotenoid and flavonoid levels were determined and compared to their wild type progenitor. In accordance to previous reports (Ronen et al., 2000), the ripe fruit (7 d.p.b) from the Og^c mutant contained comparatively higher levels of carotenoid (3-fold). This was predominantly due to an increase in lycopene [**1**] levels (Table 2). The absolute amounts of β-carotene [**2**] were reduced, but must notable was the ratio of lycopene to β-carotene where in the Og^c mutant a 20:1 ratio existed compared to a 7:1 ratio in the wild type. These findings reflect the mutation in lycopene cyclase that exists in this mutant, restricting the formation of β-carotene [**2**] from lycopene [**1**] (Ronen et al., 2000). The phenylpropanoid and flavonoid content of the Og^c mutant was unchanged compared to the background wild type (Tables 3 and 4). Relatively small fluctuations in composition were found, which presumably reflect environmental conditions rather than biochemical phenomena. The other tomato mutant within the collection possessing a mutation in a biosynthetic enzyme was the *rr*-mutant, which, with the exception of lutein [**11**], was devoid of fruit carotenoids (Table 2) (Fray and Grierson, 1993). This carotenoid profile is characteristic of a mutation in phytoene synthase-1 (*Psy*-1) which catalyses the first committed step in carotenoid formation in ripening fruit. The phenylpropanoid content of the *rr*-mutant pericarp was increased slightly (30%), predominantly due to increases in chlorogenic [**10**] and *p*-coumaric [**6**] acids (Table 3). With the exception of *p*-coumaric acid [**6**] and naringenin, which

Table 1
Mutant and transgenic tomato varieties used in this study

Genotype	Description	Mutation	Reference
Ailsa Craig	Background for all mutant and transgenic lines used	NA	NA
Og^c	Old gold crimson high lycopene containing mutant	Mutation in a fruit ripening enhanced B-gene	Ronen et al. (2000)
hp-1	High pigment mutant increased fruit colour	Mutation in the DDB1 gene-negative regulator of light signalling	Liu et al. (2004)
rr	Yellow flesh-absence of fruit carotenoids	Mutation in PSY-1	Fray and Grierson (1993)
DXS	Up-regulation of the DXS enzyme	Expression of the *E. coli* DXS under fibrillin promoter control	Enfissi et al. (2005)
LA3771	Increased lycopene content	*hp1*/Og^c hybrid	Tomato genetic resource centre
ASF5H	Ferulate [**8**] accumulation	Antisense ferulate 5-hydroxylase	Rees (2000)
CRTI	Increased β-carotene [**2**]	Expression of the *Erwinia uredovora* phytoene desaturase	Roemer et al. (2000)

NA, not applicable.

Table 2
Relative carotenoid content of ripe tomato fruit from mutant and transgenic varieties

Genotype	Carotenoid content (relative levels to Ailsa Craig background; % composition)				
	Phytoene [**12**]	Lycopene [**1**]	β-Carotene [**2**]	Lutein [**11**]	Total
Ailsa Craig	1.0 ± 0.03 (15.0)	1.0 ± 0.06 (74)	1.0 ± 0.04 (10)	1.0 ± 0.03 (1)	1.0 ± 0.04
Og^c	1.2 ± 0.1 (10.0)	2.0 ± 0.2 (85)	0.6 ± 0.11 (4)	2.2 ± 0.45 (1)	3.0 ± 0.31
hp-1	1.6 ± 0.01 (8.0)	3.4 ± 0.4 (83)	2.1 ± 0.2 (7)	2.2 ± 0.05 (1)	2.9 ± 0.35
rr	0	0	0	1.0 ± 0.005 (100)	0.01 ± 0
DXS	1.9 ± 0.4 (24.0)	1.1 ± 0.05 (67)	0.8 ± 0.1 (8)	1.7 ± 0.005 (1)	1.2 ± 0.1
LA3771	2.2 ± 0.1 (11.0)	3.5 ± 0.13 (82)	1.8 ± 0.1 (6)	4.0 ± 0.34 (1)	3.1 ± 0.1
ASF5H	1.2 ± 0.02 (10.2)	1.9 ± 0.16 (85)	0.8 ± 0.05 (6)	3.5 ± 0.03 (1)	1.7 ± 0.1
CRTI	0.1 ± 0.01 (1.4)	0.9 ± 0.04 (58)	4.3 ± 0.4 (4)	1.5 ± 0.1 (4)	1.1 ± 0.05

Fruit from three plants were harvested, pooled and six analytical replicates performed. Data represent means ± SEM. Although in some cases analysis was performed at different seasonal periods, all crops were randomised against Ailsa Craig background controls for normalisation. Typical values for Ailsa Craig phytoene, lycopene, β-carotene and lutein were 0.3–0.25, 1.4–2.5, 0.2–0.3, 0.02–0.05 mg/g DW, respectively.

Table 3
Relative phenylpropanoid and flavonoid content of pericarp tissue from ripe tomato fruit

Genotype	Phenylpropanoids and flavonoids (relative levels compared to Ailsa Craig; % composition)				
	Chlorogenic acid [**10**]	Caffeic acid [**7**]	*p*-Coumaric acid [**6**]	Ferulic acid [**8**]	Total
Ailsa Craig	1.0 ± 0.2 (25.0)	1.0 ± 0.1 (52.0)	1.0 ± 0.07 (15.0)	1.0 ± 0.15 (8.0)	1.0 ± 0.05
Og^c	0.8 ± 0.3 (19.0)	1.2 ± 0.1 (56.0)	1.6 ± 0.3 (20.0)	0.8 ± 0.09 (6.0)	1.1 ± 0.06
hp-1	4.3 ± 0.2 (45.0)	1.0 ± 0.02 (19.0)	1.5 ± 0.05 (9.0)	1.2 ± 0.08 (3.0)	2.4 ± 0.09
rr	1.4 ± 0.2 (26.0)	1.0 ± 0.1 (39.0)	2.5 ± 0.2 (28.0)	1.2 ± 0.09 (7.0)	1.3 ± 0.1
DXS	0.7 ± 0.1 (61.0)	1.1 ± 0.05 (28.0)	0.8 ± 0.1 (7.0)	1.7 ± 0.005 (4.0)	0.9 ± 0.1
LA3771	2.2 ± 0.1 (85.0)	1.1 ± 0.03 (8.0)	1.0 ± 0.04 (4.0)	1.4 ± 0.06 (3.0)	2.5 ± 0.1
ASF5H	1.9 ± 0.02 (47.0)	0.7 ± 0.06 (33.0)	0.9 ± 0.03 (13.0)	0.9 ± 0.03 (7.0)	1.0 ± 0.1
CRTI	0.6 ± 0.1 (55.0)	0.8 ± 0.14 (29.0)	1.1 ± 0.05 (8.0)	0.8 ± 0.1 (5.0)	0.83 ± 0.3

Fruit from three plants were harvested, pooled and six analytical replicates performed. Data represent mean values ± SEM. Although in some cases analysis was performed at different seasonal periods all crops were randomised against Ailsa Craig background controls for normalisation. Typical Ailsa Craig values include 0.06–0.14 μg/mg DW chlorogenic acid, 0.2–0.22 μg/mg DW caffeic acid, 0.05–0.07 μg/mg DW *p*-coumaric acid, 0.03–0.04 μg/mg DW ferulic acid. No rutin or naringenin were detected.

Table 4
Relative phenylpropanoid and flavonoid content of skin tissue from ripe tomato fruit

Genotype	Phenylpropanoids and flavonoids (relative levels compared to Ailsa Craig; % composition)						
	Chlorogenic acid [**10**]	Caffeic acid [**7**]	*p*-Coumaric acid [**6**]	Ferulic acid [**8**]	Rutin [**3**]	Naringenin	Total
Ailsa Craig	1.0 ± 0.1 (5.5)	1.0 ± 0.03 (4.0)	1.0 ± 0.03 (0.8)	1.0 ± 0.3 (1.0)	1.0 ± 0.02 (64)	1.0 ± 0.1 (25)	1.0 ± 0.02
Og^c	0.7 ± 0.1 (4.0)	0.6 ± 0.05 (2.0)	0.6 ± 0.1 (0.5)	0.4 ± 0.03 (0.5)	0.8 ± 0.1 (52)	2.0 ± 0.1 (40)	1.0 ± 0.1
hp-1	3.1 ± 0.3 (6.3)	0.8 ± 0.05 (1.0)	0.8 ± 0.06 (0.2)	0.5 ± 0.07 (0.3)	3.0 ± 0.2 (72)	2.1 ± 0.4 (21)	2.7 ± 0.3
rr	0.6 ± 0.03 (6.0)	1.1 ± 0.02 (8.0)	2.8 ± 0.1 (0.3)	1.03 ± 0.22 (0.3)	0.7 ± 0.03 (32)	1.5 ± 0.1 (62)	1.1 ± 0.1
DXS	0.1 ± 0.01 (2.0)	0.9 ± 0.08 (1.2)	0.9 ± 0.1 (0.2)	0.7 ± 0.06 (0.4)	0.4 ± 0.02 (30)	0.8 ± 0.6 (67)	0.5 ± 0.04
LA3771	2.4 ± 0.2 (9.0)	1.0 ± 0.03 (0.3)	0.9 ± 0.3 (0.1)	1.3 ± 0.2 (0.2)	0.4 ± 0.2 (73)	1.1 ± 0.1 (19)	2.5 ± 0.1
ASF5H	0.7 ± 0.14 (2.4)	0.6 ± 0.03 (1.0)	0.9 ± 0.03 (0.4)	0.3 ± 0.05 (0.3)	3.9 ± 0.1 (35)	1.1 ± 0.2 (59)	1.6 ± 0.02
CRTI	0.5 ± 0.1 (2.0)	0.8 ± 0.17 (2.0)	1.0 ± 0.16 (0.6)	0.4 ± 0.04 (0.5)	1.3 ± 0.3 (68)	1.3 ± 0.3 (27)	1.2 ± 0.2

Fruit from three plants were harvested pooled and six analytical replicates performed data represented as mean values ± SEM. Although in some cases analysis was performed at different seasonal periods all crops were randomised against Ailsa Craig background controls for normalisation. Typical Ailsa Craig values include 0.2–1.3 μg/mg DW chlorogenic acid, 0.07–0.2 μg/mg DW caffeic acid, 0.01–0.03 μg/mg DW *p*-coumaric acid, 0.02–0.6 μg/mg DW ferulic acid, 3.5–6.0 μg/mg DW rutin and 2.5–6.0 μg/mg DW naringenin.

showed significant increases in the skin, total phenylpropanoid and flavonoid content was relatively unchanged (Table 4). Therefore, it would appear that despite representing two very important classes of complementary cellular antioxidant molecules there is very little biochemical crosstalk between the two pathways. This would suggest that the carotenoid pathway can be up- or down-regulated without impacting on phenylpropanoid or flavonoid levels.

2.3. Relative consequences of mutation and transgenesis – evidence for cross-talk?

The transgenic lines with altered carotenoid contents included the DXS up-regulated plants (Enfissi et al., 2005) which contained increased carotenoids predominantly at the level of phytoene, and the CRTI line (Roemer et al., 2000), containing a bacterial (*Erwinia uredovora*) desaturase, which resulted in fruit with elevated (4.0-fold) β-carotene content and lutein levels, while phytoene [**12**] was reduced 10-fold (Table 2). In both, pericarp and skin, the phenylpropanoid and flavonoid contents were unchanged although an increased compositional change in chlorogenic acid was found in CRTI pericarp tissue (Table 3). The phenylpropanoids present in the DXS line were unchanged in the pericarp but a significant reduction of both phenylpropanoids and flavonoids was determined in the skin tissue (Table 4). Thus, the findings from the transgenic lines with altered carotenoid content are in agreement with the natural mutants in illustrating that metabolic diversity in carotenoid content does not have a significant impact on either phenylpropanoid or flavonoid content.

In order to potentially raise the levels of ferulate [**8**] in tomato fruit we generated lines that were antisense or partial sense for the cytochrome P450 ferulate 5-hydroxylase (Rees, 2000). However, the enzyme is now thought to act primarily at the level of the aldehyde and/or alcohol (Humphreys et al., 1999; Franke et al., 2000). ASF5H exhibiting reduced ferulate 5-hydroxylase expression possessed increased chlorogenic acid [**10**] but total phenylpropanoids were unchanged in pericarp tissue. In skin tissue naringenin levels were elevated (3.9-fold). The carotenoid content in this line showed increased levels, predominately due to elevated lycopene [**1**] and lutein [**11**] content compared to the Ailsa Craig background. Although limited in terms of the range of transgenic lines available these data suggest that alterations in flavonoid/phenylpropanoid content can affect carotenoid content in the fruit, whether this is a biochemical effect or results from altered ripening properties awaits elucidation.

Both the *hp*-1 and LA3771 lines showed increased carotenoid content, including phytoene, lycopene [**1**], β-carotene [**2**] and lutein [**11**] (Table 2) as well as elevated phenylpropanoids/flavonoids in the pericarp and skin tissues (Tables 3 and 4). Chlorogenic acid [**10**] was responsible for the increase in the pericarp tissue, while in skin tissue rutin [**3**] as well as chlorogenic acid [**10**] were elevated. These two natural varieties are therefore examples where both lipophilic and hydrophilic antioxidants are present in elevated levels. The *hp*-1 mutant possesses a mutant UV-damaged DNA-binding protein 1 (DDB1) gene which is a negative regulator of light signalling. Thus the plant in effect possess continuous perception of light (Liu et al., 2004). The LA3771 mutant is a hybrid between *hp*-1 and Og^c. Therefore, it would appear that manipulation of environmental sensing mechanisms are the most effective means of creating high carotenoid/flavonoid fruit. Such an approach has been developed effectively in the case of *DET*-1 (detiolated-1) down regulation. In this example, the *DET*-1 when down-regulated in a fruit specific manner yielded ripe fruit with increased carotenoids and flavonoids without affecting yield (Davuluri et al., 2005). The $hp\text{-}2^J$ and $hp\text{-}2^{dg}$ mutants possess different mutated alleles of the *DET*-1 gene and metabolic characterisation of the latter has also shown increased flavonoids and carotenoids (Bino et al., 2005). For the *hp*-1 mutant it has been postulated that increased carotenoid content is due to elevated plastid number per cell (Cookson et al., 2003). Although this is likely to be a contributing factor it is unlikely to be the sole reason behind elevated flavonoid content as they are not synthesised in the plastid. High carotenoid and flavonoid fruit have also been generated when transforming tomato with the *cry*-2 gene. This cryptochrome is a blue receptor and thus shows that it is not just components of the light signalling pathway that can confer simultaneous elevation of carotenoids and flavonoids (Giliberto et al., 2005). These various *hp* phenotypes shows the necessity for screening of mutant and metabolic diversity collections (Schauer et al., 2005; Gur and Zamir, 2004) for chemotypes with elevated health-promoting phytochemicals which have the commercial benefits of conferring consumer health based traits. In addition, LA3771 can form the basis of a background variety in which the carotenoid or flavonoid pathways can be further engineered as demonstrated previously (Bovy et al., 2002; Fraser et al., 2002).

2.4. Concluding remarks

In summary, this work describes natural and genetically modified tomato lines with both high carotenoid and flavonoid contents in ripe fruit. These lines can act as the hosts for further genetic manipulation. In addition, it would appear that perturbations to the biosynthesis of phenylpropanoids/flavonoids and carotenoids independently has no detrimental affect on the overall content of these important lipophilic and hydrophilic compounds.

3. Experimental

3.1. Standards for metabolic profiling

The crystalline reference carotenoids lycopene and β-carotene were obtained from Hoffman-La Roche (Basel, Switzerland). Lutein [**11**] was kindly provided by Kemin Industries (Des Moines, IA, USA). α-Carotene [**13**] was purchased from Sigma Chemical Co. (Poole, Dorset, UK). The acyclic carotenes phytoene [**12**], phytofluene [**14**] and ζ-carotene [**15**] were purified from the *Phycomyces blakesleeanus* S442 mutant, as described by Fraser et al. (1991). Neurosporene [**16**] was obtained from *Escherichia coli* harbouring the plasmid pACCAR-EBI (Albrecht et al., 1995) containing the phytoene desaturase gene from *Rhodobacter capsulatus*. δ-Carotene [**17**] was purified from ripe fruit of the *del* tomato. Violaxanthin [**18**], neoxanthin [**19**] and antheraxanthin [**20**] were isolated from tomato leaf tissue. Cinnamic, *p*-coumaric [**6**], caffeic [**7**], ferulic [**8**] and sinapic [**9**] acids, cholorogenic [**10**] acid, naringenin, rutin [**3**] and quercetin [**21**] were all obtained from ExtraSynthese, Lyon, France (see Fig. 1).

3.2. Tomato lines

Lycopersicon esculentum Mill. cv Ailsa Craig was used as the wild-type standard. Old gold crimson (og^c), *rr*, high pigment *hp*-1 (Harris and Spurr, 1969) and LA3771 were commercial mutant lines; *Psy*-1 (Truesdale, 1994), CRTI (Roemer et al., 2000), were previously described tomato transgenic lines. ASF5H is a tomato line antisense for ferulate 5-hydroxylase (Rees, 2000).

3.3. Growth of tomato lines

Tomato plants were grown in a greenhouse with supplementary lighting (01:00–16:00 h giving a 16 h day) with heating set points: night = 18 °C, day = 19 °C; ventilator set points: night = 20 °C, day = 21 °C. Fruit was typically harvested at 7–10 days post-breaker (i.e. red ripe). Fruit were cut in half, seeds removed and then frozen at −20 °C. Skin and pericarp tissue typically from three tomatoes was separated, freeze-dried and stored at −70 °C.

3.4. Extraction, separation and identification of carotenoids and isoprenoids

The skin and pericarp were separated from tomato fruit and freeze dried separately. Freeze-dried material (10–200 mg) was ground using a mortar and pestle. Tomato skin was also ground into fine powder using a freezer mill. Small-scale extractions were carried out in micro-centrifuge tubes (1.5 ml) or alternatively screw-capped Pyrex test tubes (15 ml). Whenever possible, all subsequent manipulations were carried out on ice and shielded from strong light. For extraction of 1–2 mg of ground freeze-dried material, MeOH (100 μl) was added, along with the internal

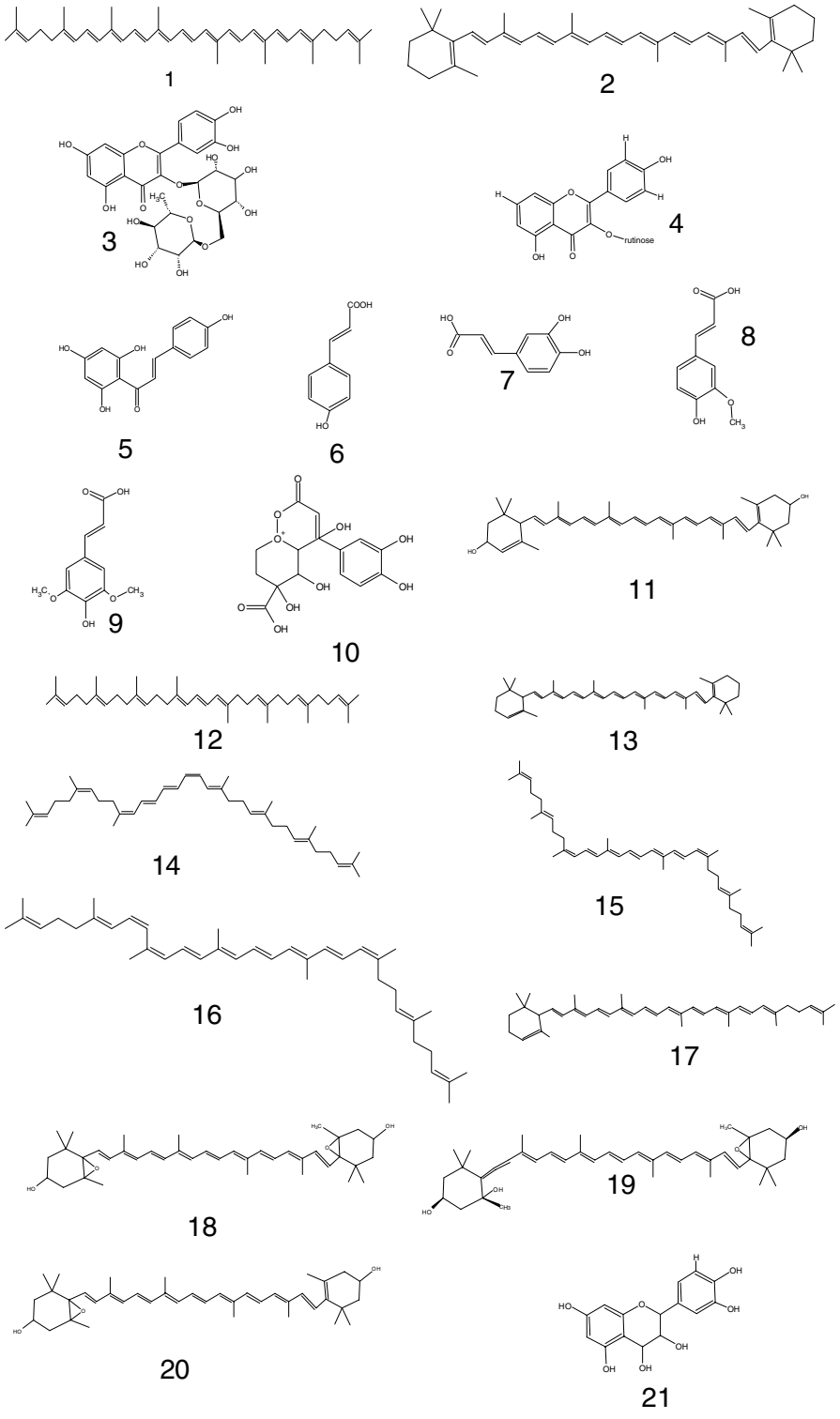

Fig. 1. Structures of compounds analysed in this study. **1**, lycopene; **2**, β-carotene; **3**, rutin; **4**, kaempferol-3-*O*-rutinoside; **5**, naringenin chalcone; **6**, *p*-coumaric acid; **7**, caffeic acid; **8**, ferulic acid; **9**, sinapic acid; **10**, chlorogenic acid; **11**, lutein; **12**, phytoene; **13**, α-carotene; **14**, phytofluene; **15**, ζ-carotene; **16**, neurosporene; **17**, δ-carotene; **18**, violaxanthin; **19**, neoxanthin; **20**, antheraxanthin; **21**, quercetin.

standard (e.g. canthaxanthin, 1 μg). The suspension was mixed by inversion for 5 min at 4 °C. Tris–HCl (50 mM, pH 7.5) (containing 1 M NaCl) was then added (100 μl) and a further incubation at 4 °C for 10 min carried out. $CHCl_3$ (400 μl) was added to the mixture and incubated on ice for 10 min. A clear partition was formed by centrifugation at 3000*g* for 5 min at 4 °C. The hypophase was removed with a Pasteur pipette and the aqueous phase re-extracted with chloroform (400 μl). The pooled $CHCl_3$ extracts were dried under a stream of nitrogen or by

centrifugal evaporation. Dried residues were stored under an atmosphere of nitrogen at −20 °C prior to HPLC. In order to efficiently extract larger quantities (e.g. fresh tissue or 50–500 mg dry powder), the volume of buffer (50 mM Tris–HCl pH 7.5) and MeOH was increased to 1.5 ml and that of $CHCl_3$ to 4 ml. If fresh tissue was used, homogenization was carried out with an Ultra-Turrax T25 (Janke and Kunkel, Staufen, Germany). When necessary, saponification was performed by adding 60% w/v KOH and MeOH to the suspension of ground tissue until a final concentration of 6% (w/v) was reached. The mixture was heated at 60 °C for 30 min in darkness. Buffer (50 mm Tris–HCl pH 7.5) was then added and the extraction performed as described above.

3.5. Isoprenoid separation and detection by HPLC–PDA

Samples were prepared for HPLC by dissolving the residues in EtoAc. Chromatography was carried out on either a Waters system (Watford, Hertfordshire, UK) consisting of a No. 616 pump, No. 996 diode array detector and No. 717 auto-sampler or a Waters Alliance 2600S system with No. 996 diode array. Data were collected and analysed using the Waters Millennium software supplied (Fraser et al., 2000). Throughout chromatography, the eluate was monitored continuously from 200 to 600 nm. Column temperature was maintained at 25 °C by a No. 7955 column oven (Jones Chromatography, Hengoed, Mid-Glamorgan, UK). A reversed-phase C_{30}, 5 μm column (250 × 4.6 mm) coupled to a 20 × 4.6 mm C_{30} guard (YMC Inc., Wilmington, NC, USA) with mobile phases consisting of MeOH (A), H_2O/MeOH (1:4 by volume) containing 0.2% ammonium acetate (B) and *tert*-methyl butyl ether (C) was also used. The gradient elution used with this column was 95% A, 5% B isocratically for 12 min, a step to 80% A, 5% B, 15% C at 12 min, followed by a linear gradient to 30% A, 5% B, 65% C by 30 min. A conditioning phase (30–60 min) was then used to return the column to the initial concentrations of A and B. In addition, a normal phase Inert Sil 5 μm, 250 × 4.6 mm column with identical guard unit (10 × 4.6 mm) purchased from Chrompak (Walton, Surrey, UK) was used. The mobile phases with this column were either 0.5% EtOH in *n*-hexane run isocratically, or gradient elution from 20% EtoAc in hexane to 100% EtoAc over 25 min, maintaining EtoAc for a further 10 min. In all cases, flow rates of 1 ml min^{-1} were used.

3.6. Phenolic profiling: extraction, separation and identification of phenolics

Freeze-dried and powdered tomato skin (20 mg) was extracted with MeOH (1 ml) containing salicylic acid as an internal standard (1 μg) in screw cap Pyrex test tubes and incubated at 90 °C for 60 min then placed on ice until cool. After centrifuging, MeOH was removed from the extracts under N_2. β-Glucosidase was added to extracts in H_2O and incubated at 37 °C for 2 h. After cooling on ice, MeOH–H_2O (1:1, v/v) was added. Extracts were filtered before HPLC analysis.

HPLC analysis was carried out on a Dionex Gynkotek system and analysed using Chromeleon software. The column temperature was maintained at 24 °C throughout chromatography. A reversed phase 5 C18 column 250 mm × 4.6 mm column (Hichrom, Reading, UK) with a 5C18 guard column (Hichrom) was used. The mobile phases consisted of: (A) 2% (aq.) MeOH containing 0.015% HCl and (B) CH_3CN. The gradient program was 95% A, 5% B for 10 min, followed by a linear gradient to 50% B. A conditioning phase over 5 min took the column back to the starting conditions, which were held for 5 min. Identification of peaks was by comparison to standard spectra and quantification was by comparison to peak areas of standards at 320 nm. Peak areas of the standards were determined at the wavelength providing maximum absorbance.

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