

AtMYB12 regulates caffeoyl quinic acid and flavonol synthesis in tomato: expression in fruit results in very high levels of both types of polyphenol

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Summary

Plant polyphenolics exhibit a broad spectrum of health-promoting effects when consumed as part of the diet, and there is considerable interest in enhancing the levels of these bioactive molecules in plants used as foods. AtMYB12 was originally identified as a flavonol-specific transcriptional activator in *Arabidopsis thaliana*, and this has been confirmed by ectopic expression in tobacco. AtMYB12 is able to induce the expression of additional target genes in tobacco, leading to the accumulation of very high levels of flavonols. When expressed in a tissue-specific manner in tomato, AtMYB12 activates the caffeoyl quinic acid biosynthetic pathway, in addition to the flavonol biosynthetic pathway, an activity which probably mirrors that of the orthologous MYB12-like protein in tomato. As a result of its broad specificity for transcriptional activation in tomato, AtMYB12 can be used to produce fruit with extremely high levels of multiple polyphenolic antioxidants. Our data indicate that transcription factors may have different specificities for target genes in different plants, which is of significance when designing strategies to improve metabolite accumulation and the anti-oxidant capacity of foods.

Keywords: transcription factor, flavonoids, caffeoyl quinic acids, secondary metabolism, metabolic engineering, polyphenols.

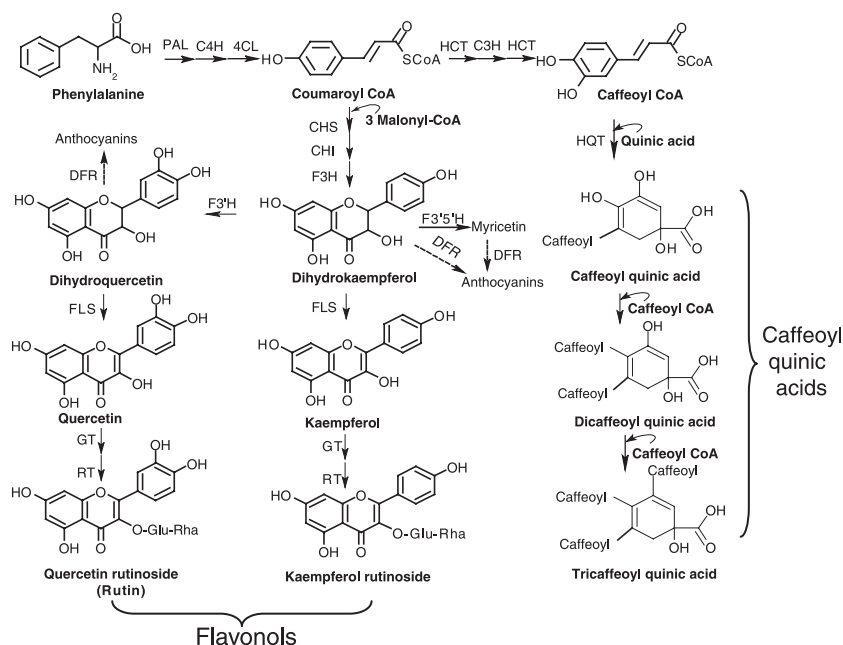
Introduction

One subclass of plant polyphenols, flavonoids, exhibits a broad spectrum of health-promoting effects (Hou *et al.*, 2004; Joseph *et al.*, 1999; Mink *et al.*, 2007; Renaud and de Lorgeril, 1992; Seeram *et al.*, 2004) when consumed in foods of plant origin. Dietary flavonoids inhibit low-density lipid oxidation and thus reduce the primary risk factor for atherosclerosis and related diseases (Brouillard *et al.*, 1997; Hannum, 2004). Longer-term dietary administration of flavonoids offers cardioprotection in rats and improves the levels of risk factors in mouse models of cardiovascular disease, although it is likely that they protect indirectly by inducing endogenous scavenging mechanisms for reactive

oxygen species (Vina *et al.*, 2007). In addition, flavonols afford protection by mechanisms beyond their activities as anti-oxidants or as inducers of scavenging pathways for reactive oxygen species, for example by inhibiting platelet aggregation and hence factors contributing to stroke and thrombosis (Nijveldt *et al.*, 2001). These data from cell-based assays and feeding trials with animals are supported by human epidemiological studies that have shown inverse correlations between consumption of flavonol-rich diets and the occurrence of cardiovascular disease, certain cancers and age-related degenerative diseases (Hertog *et al.*, 1993; Hou *et al.*, 2004; Joseph *et al.*, 1999; Renaud and de Lorgeril,

Figure 1. Phenylpropanoid biosynthesis pathway.

Only the branches that are relevant to this study are shown. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-hydroxycinnamoyl CoA ligase; 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; GT, flavonol-3-glucosyltransferase; RT, flavonol-3-glucoside-rhamnosyltransferase; C3H, *p*-coumaroyl ester 3-hydroxylase; HCT, cinnamoyl CoA shikimate/quinate transferase; HQT, hydroxycinnamoyl CoA quinate transferase.



1992; Seeram *et al.*, 2004). Based on such studies, it has been suggested that a systemic increase in the daily intake of certain flavonoids could lead to between 7 and 31% reduction in the incidence of all cancers and between 30 and 40% reduction in deaths from coronary heart disease (Hertog *et al.*, 1993; Soobrattee *et al.*, 2006).

Another important group of plant-based bioactive polyphenols are the caffeoyl quinic acids (CQAs, Figure 1), of which chlorogenic acid (CGA) is the major soluble phenolic in Solanaceous species such as potato, tomato and eggplant (aubergine), and in coffee (Clifford, 1999; Johnson and Schaal, 1952; Lepelletier *et al.*, 2007; Moores *et al.*, 1948; Niggeweg *et al.*, 2004). Consequently, CGA is one of the most abundant polyphenols in the human diet, and is the major anti-oxidant in the average US diet. CQAs have significant anti-oxidant activity and can limit low-density lipid oxidation. Other CQAs, such as dicafeoylquinic acid (diCQA) and tricafeoylquinic acid (triCQA), offer even greater protection than monocateoyl quinic acid (CGA) (Islam, 2006). Dietary CQAs are also beneficial in specific ways: consumption of triCQA reduces the blood glucose content of diabetic rats (Islam, 2006), CQAs are neuroprotective against retinal damage (Nakajima *et al.*, 2007), 3,4,5-triCQA inhibits HIV/AIDS progression (Tamura *et al.*, 2006), and diCQA has anti-hepatotoxic activity (Choi *et al.*, 2005), suggesting that CQAs offer protection by a range of mechanisms.

Plant polyphenolics have synergistic effects on health; the anti-bacterial activities of flavonoids are enhanced when they are administered in combination (Arima *et al.*, 2002), and quercetin and kaempferol inhibit cancer cell proliferation synergistically (Ackland *et al.*, 2005). Thus combinations

of flavonoids, which are present naturally in fruit and vegetables, are more effective in preventing disease than individual flavonoids.

Various strategies have been used to increase the production of polyphenolic compounds such as CQAs and flavonols in food plants. Overexpression of genes encoding individual biosynthetic enzymes can lead to modest increases in the levels of flavonols (Muir *et al.*, 2001; Niggeweg *et al.*, 2004). The problem with this type of strategy is that it does not induce the entire biosynthetic pathway, and the overall increase in end products is usually not high enough for practical application purposes. An alternative strategy involves expression of regulatory genes that directly induce these pathways. For example, increases in flavonols up to 20-fold were obtained by simultaneous expression of the maize transcription factors Lc and C1 (which induce anthocyanin biosynthesis in maize) in tomato fruit (Bovy *et al.*, 2002). Although this type of strategy generally provides a more effective means of engineering metabolism and achieves significant enhancements in the levels of end products, care is needed when choosing the transcription factor(s) to use, to ensure they have the appropriate specificity in the target crop species. For example, because Lc and C1 transcription factors were unable to induce the whole flavonol biosynthetic pathway in tomato, the overall levels of flavonol derivatives (20 times more than in the control) were still not great enough for application purposes. In addition, this strategy is problematic for pathways such as CQA biosynthesis, where regulatory proteins have not yet been identified.

We have found that it is possible to accumulate very high levels of polyphenolic compounds by tissue-specific expres-

sion of a single gene from *Arabidopsis thaliana* encoding the transcription factor AtMYB12 (Mehrtens *et al.*, 2005). Importantly, even though it was identified as a flavonol-specific transcriptional activator in Arabidopsis, a result that has been confirmed by its expression in tobacco, AtMYB12 can activate the CQA biosynthetic pathway when expressed in a tissue-specific manner in tomato, a function that probably mirrors the function of the orthologous MYB12-like protein in tomato. Consequently, AtMYB12 can be used to produce fruit with extremely high levels of multiple, health-promoting, hydrophilic, polyphenolic anti-oxidants.

Results

Effects of constitutive expression of AtMYB12 on phenylpropanoid metabolism in tobacco

AtMYB12 has been identified as a transcription factor that specifically activates flavonol accumulation in Arabidopsis (Mehrtens *et al.*, 2005). To determine whether AtMYB12 could work in the same way in other species, we introduced the AtMYB12 cDNA into tobacco (*Nicotiana tabacum*) under the control of the strong constitutive CaMV 35S promoter. Of more than 15 independent transformants, three lines (lines 10, 13 and 15) with various levels of transgene expression were investigated further. The transgenic lines grew slightly

more slowly than controls. The only other visible difference was that the flowers of the transgenic plants were more palely coloured than their wild-type counterparts (Figure 2a), and this phenotype was correlated with the expression level of the AtMYB12 transgene. The levels of phenolics in leaves and flowers of transgenic plants were compared to those in wild-type plants (Figure 2b and Table S1). Individual phenolics were identified by examination of their fragmentation patterns, and comparison, where possible, to fragmentation patterns reported in the literature. In the absence of NMR data, these identities should be considered as probable rather than proven. CQAs were by far the most abundant phenolics in wild-type leaves ($15.1 \pm 1.7 \text{ mg g}^{-1} \text{ DW}$), although low levels ($0.4 \pm 0.1 \text{ mg g}^{-1} \text{ DW}$) of quercetin glycosides (mainly rutin) were identified, but kaempferol glycosides were barely detectable. Expression of AtMYB12 resulted in 46-fold and 83-fold increases of rutin ($22.1 \pm 2.5 \text{ mg g}^{-1} \text{ DW}$) and kaempferol rutinoside ($19.2 \pm 2.1 \text{ mg g}^{-1} \text{ DW}$) in leaves, respectively. In addition, a twofold increase in CGA levels (to $36.2 \pm 4.3 \text{ mg g}^{-1} \text{ DW}$) was detected in leaves of the transgenic lines, and, in total, levels of polyphenols in excess of $77.5 \text{ mg g}^{-1} \text{ DW}$ accumulated in tobacco leaves. The increases in flavonol levels in tobacco were considerably greater than those reported for overexpression of AtMYB12 in Arabidopsis (three- to fourfold; Mehrtens *et al.*, 2005).

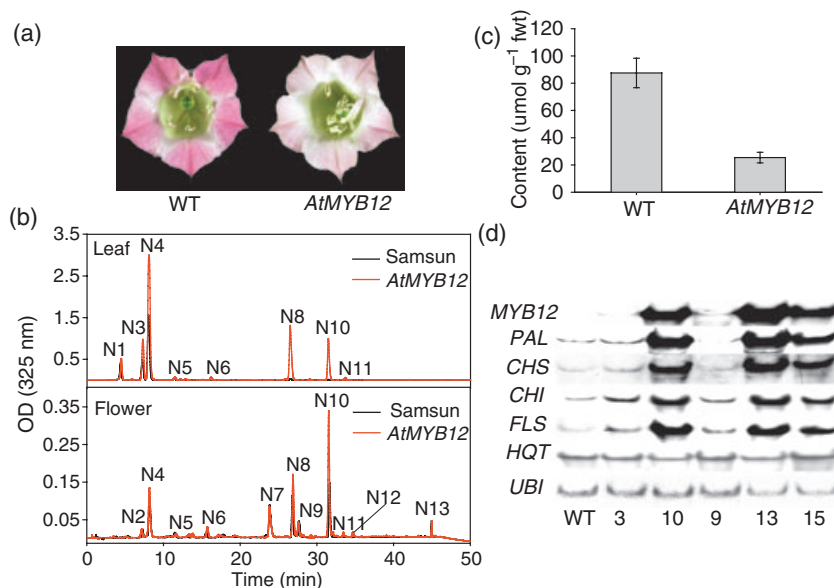


Figure 2. Expression of AtMYB12 in tobacco.

(a) Flowers of wild-type (left) and an AtMYB12-expressing line (right).

(b) HPLC analysis of methanol extracts from leaf and flowers of wild-type and AtMYB12-expressing tobacco plants. N1, 3-caffeoyl quinic acid; N2, caffeoyl-spermidine; N3, 4-caffeoyl quinic acid; N4, 5-caffeoyl quinic acid; N5, quercetin glucosyl-glucoside rhamnoside; N6, kaempferol glucosyl-glucoside rhamnoside; N7, *N*-caffeoyl-*N'*-dihydrocaffeoyl spermidine; N8, quercetin rutinoside (rutin); N9, dicaffeoyl spermidine; N10, kaempferol rutinoside; N11, kaempferol malonylglucoside; N12, unknown; N13, tricumaroyl spermidine. Identification of peaks is described in Table S1.

(c) Total anthocyanin content of wild-type and AtMYB12 flower petals.

(d) RNA gel blot showing transcript levels of structural genes involved in phenylpropanoid biosynthesis in tobacco. Genes that were analysed are described in Figure 1, except the transgene AtMYB12 and the tobacco ubiquitin gene (*UBI*), which was used as a control. fwt, fresh weight.

Flowers of tobacco expressing *AtMYB12* also had increased rutin, kaempferol rutinoside, CGA and kaempferol malonylglucoside contents compared to wild-type flowers, and lower levels of anthocyanins (Figure 2c), whereas the levels of polyamine conjugates remained unchanged. The inverse correlation between anthocyanin and flavonol levels in flowers reflects competition between these two branches of flavonoid metabolism; competition that is believed to occur through the relative activities of dihydroflavonol 4-reductase and flavonol synthase on dihydroflavonol precursors (Davies *et al.*, 2003).

Effects of *AtMYB12* on gene expression in tobacco

Expression of the genes encoding enzymes involved in flavonol and CGA biosynthesis was compared between control plants and lines expressing *AtMYB12* by RNA gel blots (Figure 2d). Among the genes that were tested, those encoding phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI) and flavonol synthase (FLS) were induced by *AtMYB12* expression in tobacco. The gene encoding hydroxycinnamoyl CoA quinate transferase (HQT), which is involved in CGA biosynthesis (Niggeweg *et al.*, 2004), was not induced, and expression of the gene encoding dihydroflavonol 4-reductase (DFR), which is required specifically for anthocyanin biosynthesis, was not detected in either the control or the *AtMYB12*-expressing plants. These results confirm the genes originally identified as targets of *AtMYB12* (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007), except that the genes encoding PAL were not identified as targets of *AtMYB12* in *Arabidopsis*.

Effects of fruit-specific expression of *AtMYB12* in tomato

Our results show that *AtMYB12* can act as a very effective, positive regulator of the flavonol biosynthetic pathway in tobacco. To exploit the potential of *AtMYB12* for metabolic engineering, the gene was then introduced into tomato, driven by the fruit-specific E8 promoter, which is most active in developing fruit after the breaker stage (Deikman *et al.*, 1992). Two varieties of tomato (*Solanum lycopersicum* cv. MicroTom and cv. Money Maker) were transformed with pSLJ-E8-MYB12, and more than 30 independent kanamycin-resistant plants (T_0) were obtained for each variety.

AtMYB12 primary transformants developed normally during vegetative growth and were indistinguishable from controls. Transgenic fruit also developed normally and were indistinguishable from fruit of control plants until the turning stage. At maturity, instead of turning the pink-red colour of control fruit, the transgenic MicroTom fruit were orange (Figure 3a). This phenotype correlated with the expression level of *AtMYB12* and the content of flavonol derivatives in the transgenic fruit. The same orange fruit

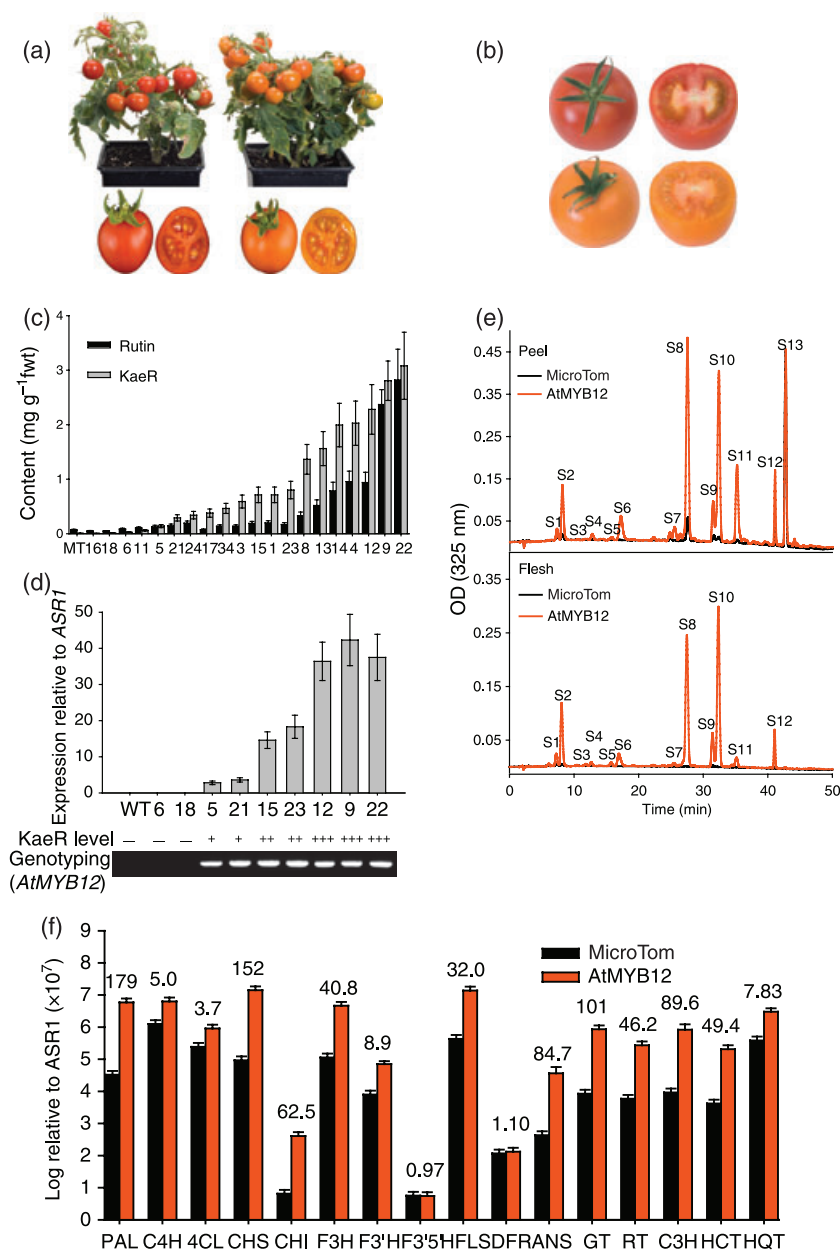
phenotype was observed in the fruit of the transformants in the Money Maker background (Figure 3b).

Mature T_0 fruits of each variety were screened for their rutin and kaempferol rutinoside contents (Figures 3c and Figure S1 for MicroTom and Money Maker, respectively). Because of the shorter generation time and relative ease of cultivation of dwarf varieties, transgenic plants in the MicroTom background with various levels of flavonol derivatives were selected for further genotyping, and the transcript levels of *AtMYB12* in the fruit of these plants were analysed by quantitative RT-PCR (Figure 3d). Control fruit showed no *AtMYB12* transcript, while all transgenic plants tested showed *AtMYB12* transcripts in fruit and all the transgenic lines expressing *AtMYB12* produced orange fruit as opposed to the normal red wild-type fruit. Furthermore, there was a good correlation between the transcript levels of *AtMYB12* and the kaempferol rutinoside contents of the fruit. Three of the transgenic plants (lines 9, 12 and 22) were used for further analyses. For all three lines, the transgene was inherited in a 3:1 ratio by the T_1 progeny, as expected for single-copy transgene insertions.

Effects of *AtMYB12* on phenylpropanoid metabolism in tomato

Ripe fruit from both transgenic and control lines were harvested, and the peel and flesh were analysed separately for their polyphenol contents by HPLC and LC/MS/MS (Figure 3e and Table S2). The main flavonoid in control peel extract was naringenin chalcone, with small amounts of rutin, kaempferol rutinoside, CGA, diCQA and triCQA (Figure 3e, Table S2 and Figure S2), confirming results reported recently by Moco *et al.* (2006). The flesh of control plants contained only small amounts of CQAs and other phenolics. In both the peel and the flesh of transgenic fruit, massive levels of flavonol derivatives (mainly rutin and kaempferol rutinoside) were detected. Surprisingly, high levels of CQAs (CGA, diCQA and triCQA) were also detected in the fruit of the lines expressing *AtMYB12* (Figure 3e). The major phenolics in whole fruit were quantified using purified standards as shown in Table 1. On a whole-fruit basis, more than 15 mg g⁻¹ DW of CQAs and 72 mg g⁻¹ DW of flavonols were detected in the transgenic fruit in the MicroTom background, which is equivalent to 22- and 65-fold higher levels, respectively, compared to control fruit. In the Money Maker background, *AtMYB12* also increased the levels of both CQAs and flavonols (to more than 3 and 48 mg g⁻¹ DW, respectively). In untransformed Money Maker, the total levels of these polyphenols were lower than in MicroTom, a feature that is common to larger-fruited varieties compared to cherry tomatoes (Raffo *et al.*, 2002).

The high-polyphenol phenotype was maintained in mature fruit of hemizygous T_1 and homozygous T_2 individuals of three single-copy *AtMYB12* lines (Table 2), showing



that the high-flavonol/high-CQA phenotype was inherited stably in subsequent generations and was in fact somewhat enhanced as the *AtMYB12* transgene was brought to homozygosity.

Effects of *AtMYB12* on gene expression in tomato

The effect of *AtMYB12* on the expression of genes involved in flavonoid biosynthesis was examined by quantitative RT-PCR in fruit from the T_1 generation of plants. RNA was extracted from fruit at the turning stage. Expression levels of phenylpropanoid pathway genes encoding PAL, cinnamate 4-hydroxylase (C4H), 4-hydroxycinnamoyl CoA ligase (4CL),

CHS, CHI, flavanone-3-hydroxylase (F3H), flavonoid-3'-hydroxylase (F3'H), flavonoid-3'5'-hydroxylase (F3'5'H), FLS, DFR, anthocyanidin synthase (ANS), flavonol-3-glucosyltransferase (GT), flavonol-3-glucoside-rhamnosyltransferase (RT), *p*-coumaroyl ester 3-hydroxylase (C3H), hydroxycinnamoyl CoA shikimate/quinic acid transferase (HCT) and HQT were compared in control and transgenic fruit expressing *AtMYB12*. Transcripts of *PAL*, *F3H*, *F3'H*, *FLS*, *GT* and *RT* genes were readily detectable in control fruit (Figure 3f). In transgenic *AtMYB12* fruit, we observed more than 100-fold induction of the genes encoding *PAL*, *CHS* and *GT*, between 50- and 100-fold induction of the genes encoding *CHI*, *ANS* and *C3H*, between 10- and 50-fold induction of the

Table 1 Quantification of major phenolics in wild-type [MicroTom (MT) and Money Maker (MM)] and AtMYB12-expressing tomatoes

Compound	MT (mg g ⁻¹ DW)	MT-MYB12 (mg g ⁻¹ DW)	Fold increase	MM (mg g ⁻¹ DW)	MM-MYB12 (mg g ⁻¹ DW)	Fold increase
CGA	0.25 ± 0.04	4.96 ± 0.72	19.8	0.04 ± 0.01	1.17 ± 0.25	27.2
diCQA	0.19 ± 0.04	4.21 ± 0.69	22.2	0.03 ± 0.01	0.85 ± 0.14	26.6
triCQA	0.28 ± 0.05	6.59 ± 1.09	23.5	0.04 ± 0.01	1.36 ± 0.28	42.5
QueRut	0.92 ± 0.20	30.90 ± 5.21	33.6	0.30 ± 0.05	20.20 ± 3.40	67.3
KaeRut	0.20 ± 0.04	41.70 ± 6.74	209.0	0.05 ± 0.01	28.50 ± 5.62	593.5
KaeGRG	ND	4.47 ± 0.64	–	ND	1.12 ± 0.21	–
NCG	ND	1.55 ± 0.27	–	ND	0.43 ± 0.08	–
NC	0.89 ± 0.24	0.85 ± 0.22	1.0	0.03 ± 0.01	0.03 ± 0.01	0.9

CGA, chlorogenic acid; diCQA, dicaffeoyl quinic acid; triCQA, tricaffeoyl quinic acid; QueRut, quercetin rutinoside; KaeRut, kaempferol rutinoside; KGRG, kaempferol glucosyl-rhamnosylglucoside; NCG, naringenin chalcone glucoside; NC, naringenin chalcone; ND, not detected.

Table 2 Stability of the AtMYB12 expression phenotype in T₁ and T₂ generations of transgenic lines of MicroTom^a

Line	Caffeoyl quinic acids (mg g ⁻¹ DW)	Quercetin rutinoside (mg g ⁻¹ DW)	Kaempferol derivatives ^b (mg g ⁻¹ DW)
T₁ generation			
MT	0.8 ± 0.1	1.0 ± 0.1	0.2 ± 0.1
Line 22-1	20.3 ± 2.9	34.9 ± 4.4	46.0 ± 6.9
Line 12-1	18.8 ± 2.6	21.9 ± 3.6	31.0 ± 4.1
Line 9-1	16.1 ± 2.1	18.8 ± 2.7	35.1 ± 4.3
T₂ generation			
MT	0.7 ± 0.1	1.0 ± 0.1	0.3 ± 0.1
Line 22-2	16.8 ± 2.0	40.5 ± 5.0	50.4 ± 6.3
Line 12-2	15.5 ± 1.7	35.4 ± 4.1	42.7 ± 5.1
Line 9-2	20.8 ± 2.2	32.6 ± 4.0	46.9 ± 5.4

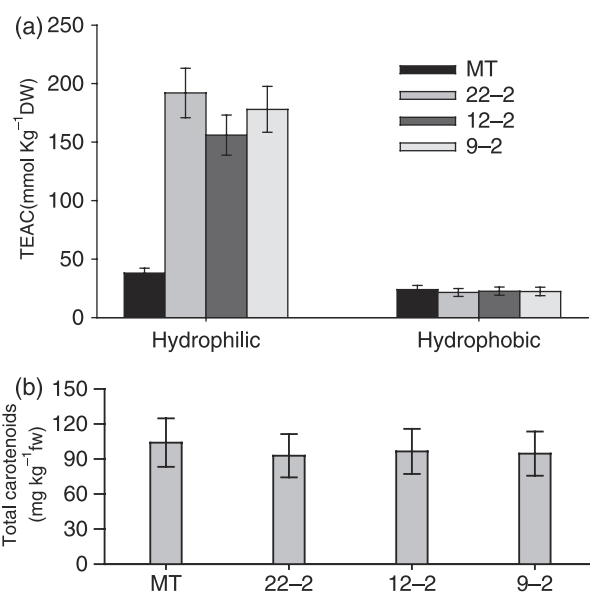
^aMature fruits were harvested from hemizygous T₁ and homozygous T₂ populations of three independent transgenic lines (lines 22, 12 and 9). Eight plants from each independent line were analysed. From each plant, two or three fruits were pooled, methanol extracts were prepared, and phenylpropanoid levels were determined. DW, dry weight.

^bTotal amount of kaempferol rutinoside and kaempferol glucosyl-rhamnosylglucoside.

genes encoding FLS, F3H, RT and HCT, and between 3- and 10-fold induction of the genes encoding F3'H, C4H, 4CL and HQT relative to the levels in wild-type fruit. Neither *F3'5'H* nor *DFR* were upregulated in AtMYB12 fruit. These results show that fruit-specific expression of AtMYB12 in tomato leads to induction of all of the biosynthetic genes required for the production of flavonols and their derivatives, and, in addition, those required for the synthesis of CGA and its derivatives.

Effects of elevated flavonol and CQA levels on the anti-oxidant capacity of tomato fruit

Differences in the total anti-oxidant capacity between transgenic and control tomato fruit were measured using the Trolox equivalent anti-oxidant capacity (TEAC) assay.

**Figure 4.** Total anti-oxidant capacity and total carotenoids in wild-type (MT) and T₂ generation AtMYB12-expressing tomato fruit.

(a) Hydrophilic and lipophilic anti-oxidant activities in mature tomato fruit from wild-type (MT) and AtMYB12-expressing lines.

(b) Total carotenoid levels in mature tomato fruit from wild-type (MT) and AtMYB12-expressing lines.

In AtMYB12 fruit, the TEAC activity of the water-soluble fraction (containing phenolics) was increased up to five-fold compared to the control (Figure 4a), but no significant difference could be detected for the TEAC activities of the lipophilic fraction between control and transgenic fruit. This suggested that the high levels of phenylpropanoids were not achieved at the expense of carotenoid accumulation in tomato fruit. No significant difference in total carotenoid content was detected between transgenic and control fruit, and the colour of the lipophilic carotenoid extracts was indistinguishable between wild-type and transgenic plants (Figure 4b). This indicates that the orange colour of the transgenic fruit is due to the accu-

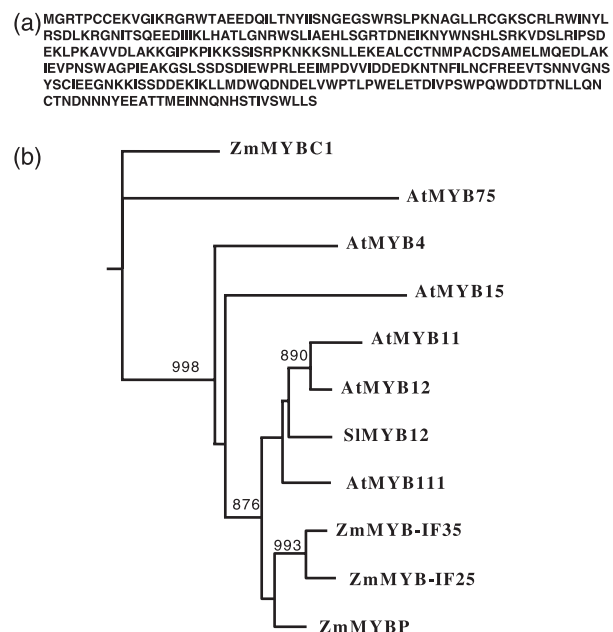


Figure 5. SIMYB12 of tomato.

(a) Amino acid sequences of SIMYB12.

(b) Alignment of SIMYB12 with other members of the R2R3MYB subgroup 7 transcription factors that regulate flavonol or phlobaphene production in plants. One thousand bootstrapped data sets were used to indicate the confidence of each tree clade. Clades supported by more than 700 out of 1000 bootstrap datasets are indicated on the tree. The following protein sequences were used for the analysis: ZmMYBC1 (AAK09327), AtMYB75 (ABB03879), AtMYB4 (AAC83582), AtMYB15 (NP_188966), AtMYB11 (AAC83585), AtMYB12 (AAC83586), SIMYB12 (EU419748), AtMYB111 (NP_190199), ZmMYB-IF35 (AAO48737), ZmMYB-IF25 (AAO48738), ZmMYBP (P27898).

mulation of flavonols rather than reductions in lycopene levels.

Function of the orthologous MYB12-like protein of tomato

To investigate the role of AtMYB12-like transcription factors in tomato, three ESTs encoding various MYB12-like proteins were identified from the tomato EST database. One, which showed the greatest sequence similarity to AtMYB12, was expressed early during fruit development. This EST sequence was used to identify a full-length cDNA clone that encodes a protein very similar to AtMYB12, which we named SIMYB12 (*Solanum lycopersicum* MYB12; Figure 5a,b). Expression of the gene encoding SIMYB12 was analysed by quantitative RT-PCR and compared to the levels of flavonols and CQAs at the same stages of fruit development (Figure 6a,b). The transcript levels increased markedly between the green and green-yellow stages of fruit development, a time at which the levels of both flavonols and CQAs also rose sharply. At later stages of ripening, the transcript levels did not increase further (and in fact declined slightly). The levels of CQAs and flavonols also increased no further, but instead declined in red tomatoes. SIMYB12 was expressed most highly in the peel of developing fruit, but was also expressed at a lower level in the flesh where lower amounts of CQAs and flavonols accumulate (Figure 6c,d). Taken together, these data suggest that regulation of CQA biosynthesis is a function of SIMYB12 in tomato fruit, in addition to its role in the regulation of flavonol biosynthesis.

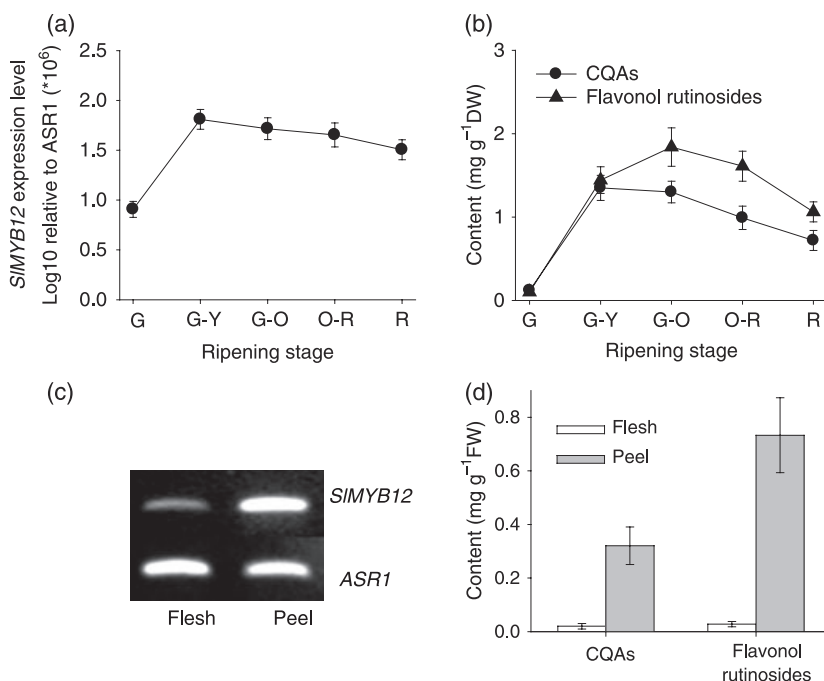


Figure 6. Polyphenolic contents and SIMYB12 transcript levels in developing MicroTom tomato fruit.

(a) SIMYB12 transcript levels in tomato fruit at various ripening stages. Transcript levels determined by quantitative RT-PCR are expressed relative to ASR1 using a log₁₀ scale.

(b) CQAs and flavonol rutinoides levels in MicroTom tomato fruit at various ripening stages.

G, green; G-Y, green-yellow; Y-O, yellow-orange; O-R, orange-red; R, red.

Results are mean $\pm$ SD from three measurements of two independent biological replicates.

(c) RT-PCR of SIMYB12 transcript levels in peel and flesh of tomato fruit at the green-yellow developmental stage. ASR1 was used as a control to indicate equal loading of template.

(d) Levels of CQAs and flavonols in flesh and peel of mature fruit of MicroTom.

Discussion

Our study demonstrates that expression of the transcription factor AtMYB12 results in unprecedentedly high levels of flavonol accumulation in both tobacco and tomato (up to about 8% of the dry weight of tobacco leaves and up to about 10% of the dry weight in whole tomato fruit).

When *AtMYB12* was expressed at high levels in tobacco, significant increases of two flavonols only, rutin and kaempferol glycoside, were observed. This ability of AtMYB12 to stimulate flavonol accumulation was predicted from analysis of AtMYB12 function in Arabidopsis, although the three- to fourfold increases induced by *AtMYB12* overexpression in Arabidopsis were significantly lower than the increases achieved in either tobacco or tomato (Mehrtens *et al.*, 2005). The large increases in flavonol accumulation in tobacco and tomato may result from the induction of *PAL* gene expression by AtMYB12 in these species; *PAL* genes were not identified as targets of AtMYB12 or other subgroup 7 transcription factors in Arabidopsis (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007). *PAL* activity has been reported to limit flux through phenylpropanoid metabolism in tobacco (Howles *et al.*, 1996), and may be key to the efficacy of AtMYB12 in tomato and tobacco. The effect of AtMYB12 on CQA levels in tobacco leaves was relatively modest. It is very likely that the twofold increase in CQA levels observed in tobacco expressing *AtMYB12* resulted from the transcription factor stimulating the expression of genes encoding *PAL*, rather than any direct effect on CQA biosynthesis, as no stimulation of the transcript levels for the genes encoding *HQT* nor *C3H* (Figure 2d and J.L. and C.M., unpublished results) occurred in tobacco. In fact, increases in CGA levels of between two- and threefold have been reported for overexpression of *PAL* in tobacco (Howles *et al.*, 1996); the same order of magnitude of increase as observed with *AtMYB12* in tobacco.

However, in tomato fruit, significantly higher levels of CQAs accumulated as a result of expression of *AtMYB12*, in addition to very high levels of flavonols. In ripe fruit, there were up to 70-fold higher levels of total flavonols and 20-fold higher levels of CQAs, compared to controls. The increased levels of both types of polyphenol were not variety-specific, and resulted in up to fivefold enhancement of the total hydrophilic anti-oxidant capacity of fruit. This is higher than previous attempts to increase anti-oxidant capacity in tomato (Giovinazzo *et al.*, 2005; Rein *et al.*, 2006).

The very significant increases in levels of polyphenols from various branches of the phenylpropanoid pathway in tomato fruit was due to the increases in transcript levels of the genes encoding enzymes of both flavonoid and CQA biosynthesis. Expression of the gene encoding *PAL* was induced more than 100-fold by AtMYB12. *PAL* has been suggested to be a major control point determining the flux into phenylpropanoid metabolism (Howles *et al.*, 1996), and

its induction is likely to be crucial to achieving high levels of accumulation of any polyphenolics. Induction of expression of at least one gene encoding *PAL* by AtMYB12 represents a significant difference between the effects of AtMYB12 and the effects of *Lc* and *C1* transcription factors from maize, which did not induce *PAL* expression in tomato (Bovy *et al.*, 2002). *Lc* and *C1* increased flavonol levels to about 0.13 mg g⁻¹ FW of tomato, whereas AtMYB12 increased flavonol levels to over 90 mg g⁻¹ DW (equivalent to 7.1 mg g⁻¹ FW) and enhanced the already high levels of CQAs by up to 20-fold (total 20 mg g⁻¹ DW, equivalent to 1.6 mg g⁻¹ FW). In addition, AtMYB12 induced the production of both quercetin and kaempferol-type flavonols. This is in contrast to the activity of *Lc* and *C1* in tomato, which primarily induced the production of kaempferol derivatives (Bovy *et al.*, 2002). This difference is attributable to activation of the gene encoding *F3'H*, which is necessary for the production of quercetin (Figure 1), by AtMYB12; this gene was not induced by *Lc* and *C1*. Interestingly, in Arabidopsis, AtMYB12 does not appear to induce *F3'H* gene expression strongly (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007). Our data indicate the importance of determining the specificity of transcription factors for their target genes in different plant species. Clearly, MYB12-like proteins differ in their target genes and hence in their quantitative and qualitative effects on metabolic flux, even in closely related species such as tobacco and tomato.

Identified originally as a flavonol-specific transcription factor in Arabidopsis, AtMYB12 has been shown to target primarily flavonol-specific genes (Stracke *et al.*, 2007). Our results show that overexpression of *AtMYB12* in tomato results in induction of not only the above-mentioned genes but also all other genes that are involved in the biosynthesis of flavonol derivatives, including those encoding *PAL*, *C4H* and *4CL*. In addition, AtMYB12 also induces increases in the transcript levels of genes involved in CQA biosynthesis, including *HCT*, *C3H* and *HQT*, in tomato. The increased accumulation of both diCQA and triCQA, which are the products of further steps in the CQA biosynthetic pathway, suggests that the additional gene(s) required for the biosynthesis of these compounds are probably also induced by expression of *AtMYB12*, although these genes remain to be identified at the molecular level.

We do not have a ready explanation for why AtMYB12 might induce gene expression in both CQA and flavonol biosynthetic pathways in tomato but only the flavonol biosynthetic pathway in tobacco. AtMYB12 does not induce CQA biosynthesis in Arabidopsis, but Arabidopsis lacks *HQT*, one of the genes required for CQA production (Niggeweg *et al.*, 2004). Interestingly, however, the *P* gene of maize and a close homologue (*IF35*), which encode transcription factors that are closely related structurally to AtMYB12 (Figure 5a), and share some of its target genes, appear to regulate the production of CGA in maize (Zhang

et al., 2003), although no data are available for the effect of P on transcript levels of the CGA biosynthetic genes in maize. *P* lies at a major QTL determining CGA levels in the silks of this crop (Szalma *et al.*, 2005), and *IF35* maps closely to a minor QTL for the same trait. These observations, together with our results regarding the effects of AtMYB12 on CQA levels in tomato fruit, suggest that regulation of the pathways for CQA biosynthesis may be an additional and perhaps ancient function of AtMYB12-like transcription factors. In tobacco, it would appear that MYB12-like proteins no longer have the capacity to activate transcription of the genes encoding C3H and HQT, and that alternative regulatory mechanisms have evolved to influence CQA accumulation. However, the fact that AtMYB12 and probably the product of the endogenous *MYB12*-like gene, SIMYB12, regulate both flavonol and CQA production and *PAL* gene expression in tomato, makes R2R3MYB transcription factors of subgroup 7 powerful tools for metabolic engineering and improving the health-promoting properties of foods, particularly in species that maintain both flavonol and CQA biosynthetic pathways. This expansion of understanding of the regulatory functions of MYB12-like transcription factors means that they may be responsible for inducing a broad range of polyphenolic compounds in various plant species.

Our use of AtMYB12 resulted in accumulation of exceptionally high levels of polyphenols in engineered plants; polyphenols collectively reached up to 8% of the dry weight of tobacco leaves and 10% of the dry weight of tomato fruit. These levels may represent the upper limits for polyphenol accumulation in viable plant tissues. Although we did observe a slightly reduced rate of growth in AtMYB12-expressing tobacco plants compared to controls, presumably as a result of the conflicting demands of primary metabolism and enhanced secondary metabolism, higher levels of polyphenol accumulation were achieved in tomato without any observed effects on fruit growth or yield. This is probably attributable to our use of the E8 promoter to drive AtMYB12 expression in tomato. Induction of high rates of flux to polyphenols specifically during the later stages of fruit ripening appeared to have no impact on growth or productivity of these terminally differentiated tissues.

Experimental procedures

Plasmid construction, plant transformation and confirmation of transgenic lines

Tobacco transformation pBin-35S-AtMYB12 was produced by cloning the AtMYB12 cDNA between the double CaMV 35S promoter and the CaMV terminator in pBin19. Tobacco (*N. tabacum* var. Samsun) was transformed with pBin-35S-AtMYB12 using *Agrobacterium tumefaciens* (LBA4404). T-DNA insertions were confirmed by PCR of genomic DNA. AtMYB12 transcript levels were measured by RNA gel blots using the tobacco ubiquitin gene as a control.

Tomato transformation The binary vector pSLJ-E8-MYB12 was constructed by replacing the double 35S promoter in pJIT160 (Guerineau and Mullineaux, 1993) by the E8 promoter to create pJIT160-E8. The full-length sequence of the AtMYB12 cDNA was amplified and inserted in pJIT160-E8 to produce pJIT160-E8-MYB12. The E8-MYB12-CaMVTerm fragment of pJIT160-E8-MYB12 was inserted into pSLJ7291 to produce pSLJ-E8-MYB12. Tomato varieties MicroTom and Money Maker were transformed by *Agrobacterium*-mediated transformation (GV3101) of cotyledons (Fillatti *et al.*, 1987). T-DNA insertions were confirmed by PCR of genomic DNA.

Analysis and identification of phenylpropanoids

Phenylpropanoids were extracted either from fresh samples using 100% methanol or from freeze-dried samples using 70% methanol. Phenylpropanoids were analysed and identified by HPLC and LC/MS/MS (Luo *et al.*, 2007). Phenylpropanoids were quantified by calculating the area of each individual peak and comparing this to standard curves obtained from the pure compounds. Pure flavonoids, kaempferol rutinoside, naringenin, naringenin chalcone were purchased from Apin Chemicals Ltd (<http://www.apinchemicals.com>) or Extrasynthese (<http://www.extrasynthese.com>). Quercetin rutinoside (rutin), chlorogenic acid (CGA) and lycopene were purchased from Sigma (<http://www.sigmaaldrich.com/>). Total anthocyanins were quantified as previously described (Martin *et al.*, 1985).

Quantitative RT-PCR

Total RNA was obtained using an RNeasy plant mini kit (Qiagen, <http://www.qiagen.com/>). First-strand cDNA was synthesized from 5 µg of total RNA using the adaptor oligo(dT)₁₇ primer (Frohman *et al.*, 1988) (Sigma) and SuperScript III (Invitrogen, <http://www.invitrogen.com/>). Quantitative real-time RT-PCR was performed using gene-specific primers as shown in Table S3, using procedures described previously (Luo *et al.*, 2007). All quantifications were normalized to *abscisic stress ripening gene 1* (*ASR1*) amplified under the same conditions using primers 5'-CCTGTTCCACCACAA-GGACAA-3' and 5'-GTGCCAAGTTTACCGATTTC-3'.

RNA gel-blot analysis

Total RNA was purified from tobacco leaves using TRI Reagent (Sigma) according to procedures provided by the manufacturer. Total RNA (20 µg) was then separated on denaturing agarose, transferred onto nylon membranes (GE Healthcare, <http://www.gehealthcare.com>), and hybridized to radioactive DNA probes. RNA gel blots were probed with labelled cDNA fragments amplified from tobacco: *PALA* (AB008199), *CHS* (AF311783), *FLS* (AB289451), *HQT* (AJ582651) and *UBI* (X77456). Hybridization was performed out as previously described (Martin *et al.*, 1991; Niggeweg *et al.*, 2004), and filters were washed three times for 15 mins each wash with 0.1× SSC, 0.5% SDS at 65°C.

Cloning of SIMYB12 and assaying its expression in tomato fruit

The tomato EST database was searched for sequences homologous to AtMYB12, and the one with the highest sequence similarity to AtMYB12 was used to identify a full-length cDNA from tomato fruit using 3' RACE PCR (Frohman *et al.*, 1988). Total RNA was isolated and first-strand cDNA was synthesized (Luo *et al.*, 2007). The 3' end of the cDNA was amplified using the oligonucleotide 5'-ATG-

GGAAGAACACCTTGTG-3' and the 3' adaptor sequence 5'-GAC-TCGAGTCGACATCG-3' (Frohman *et al.*, 1988). The amplified sequence was cloned into pGEM-T Easy (Promega, <http://www.promega.com>) and sequenced. The full-length cDNA was then re-amplified using the forward primer 5'-ATGGGAAGAACACCTTGTG-3' and the reverse primer 5'-CTAAGACAAAAGCCAAGAT-ACAA-3', based on the 3' sequence amplified by 3' RACE. The sequence for *SlMYB12* was submitted to the EMBL database with the accession number EU419748. Expression of *SlMYB12* in MicroTom tomato fruit was assayed by quantitative RT-PCR (Luo *et al.*, 2007) using primers 5'-GAGCAATAATGTAGGGAATAG-3' and 5'-TTGAA-GTAAGTTAGTGTCAGTAT-3'.

Phylogenetic analysis

Amino acid sequences were aligned using the CLUSTAL W program (Thompson *et al.*, 1994). Phylogenetic analysis was performed using the PHYLIP programs (<http://www.evolution.genetics.washington.edu/phylic.html>) (version 3.67) using the region of the alignment corresponding to the MYB DNA binding domain (indicated by 'equals' symbols). A distance-matrix method employing the Jones-Taylor-Thornton model (Jones *et al.*, 1992) was used to compare the sequences, and a tree was derived using the neighbour-joining clustering method (Saitou and Nei, 1987). One thousand bootstrapped data sets were used to indicate the confidence of each tree clade.

Total anti-oxidant activity

Freeze-dried tomato samples (50 mg) were extracted with water and then re-extracted with acetone. Water and acetone extracts were analysed for their anti-oxidant capacity. The Trolox equivalent anti-oxidant capacity (TEAC), based on the ability of anti-oxidant molecules to quench the long-lived ABTS radical cation [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate); Sigma], compared with that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma), a water-soluble vitamin E analogue, was determined (Pellegrini *et al.*, 1999). Results were expressed as the TEAC in mmol of Trolox per kg of dry weight.

Analysis of total carotenoids

Total carotenoids were obtained from freeze-dried tomato fruit samples by extraction in the dark with tetrahydrofuran (two times) for 20 min followed by centrifugation (10 000 g) at room temperature for 10 min. The supernatants were combined and absorbance was measured at 472 nm (Levin *et al.*, 2003). Quantification of total carotenoid levels was performed using a calibration curve obtained for the pure compound, lycopene.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Flavonoid levels in tomato fruits.

Figure S2. LC/MS/MS fragmentation of diCQA and triCQA.

Table S1. Identification of major phenylpropanoids in tobacco plants.

Table S2. Identification of major phenylpropanoids in tomato fruit.

Table S3. Primers used for real-time RT-PCR.

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