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Introduction of sense constructs of cinnamate 4-hydroxylase (CYP73A24) in transgenic tomato plants shows opposite effects on flux into stem lignin and fruit flavonoids

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Abstract

Understanding regulation of phenolic metabolism underpins attempts to engineer plants for diverse properties such as increased levels of antioxidant flavonoids for dietary improvements or reduction of lignin for improvements to fibre resources for industrial use. Previous attempts to alter phenolic metabolism at the level of the second enzyme of the pathway, cinnamate 4-hydroxylase have employed antisense expression of heterologous sequences in tobacco. The present study describes the consequences of homologous sense expression of tomato CYP73A24 on the lignin content of stems and the flavonoid content of fruits. An extensive number of lines were produced and displayed four developmental variants besides a normal phenotype. These aberrant phenotypes were classified as dwarf plants, plants with distorted (curly) leaves, plants with long internodes and plants with thickened waxy leaves. Nevertheless, some of the lines showed the desired increase in the level of rutin and naringenin in fruit in a normal phenotype background. However this could not be correlated directly to increased levels of PAL and C4H expression as other lines showed less accumulation, although all lines tested showed increases in leaf chlorogenic acid which is typical of Solanaceous plants when engineered in the phenylpropanoid pathway. Almost all transgenic lines analysed showed a considerable reduction in stem lignin and in the lines that were specifically examined, this was correlated with partial sense suppression of C4H. Although not the primary purpose of the study, these reductions in lignin were amongst the greatest seen in plants modified for lignin by manipulation of structural genes. The lignin showed higher syringyl to coniferyl monomeric content contrary to that previously seen in tobacco engineered for downregulation of cinnamate 4-hydroxylase. These outcomes are consistent with placing CYP73A24 more in the lignin pathway and having a role in flux control, while more complex regulatory processes are likely to be involved in flavonoid and chlorogenic acid accumulation.

Keywords: *Lycopersicon esculentum*; Solanaceae; Tomato; Flavonoids; Lignin; Cinnamate 4-hydroxylase; Sense expression; Carotenoids

1. Introduction

Tomato fruit has been a target for genetic manipulation to optimise its nutritional qualities in a number of studies. The fruit is a major dietary source of the antioxidant and anticancer agent lycopene (**1**) (Fig. 1) and of the radical scavenger β-carotene (**2**) and these compounds have been the subject of numerous attempts to increase their levels in mutant and transgenic lines (Bramley,

Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate ligase; C3′H, *p*-coumaroyl 3′-hydroxylase; F5H, ferulate 5-hydroxylase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; CHS, chalcone synthase; FLS, flavonol synthase.

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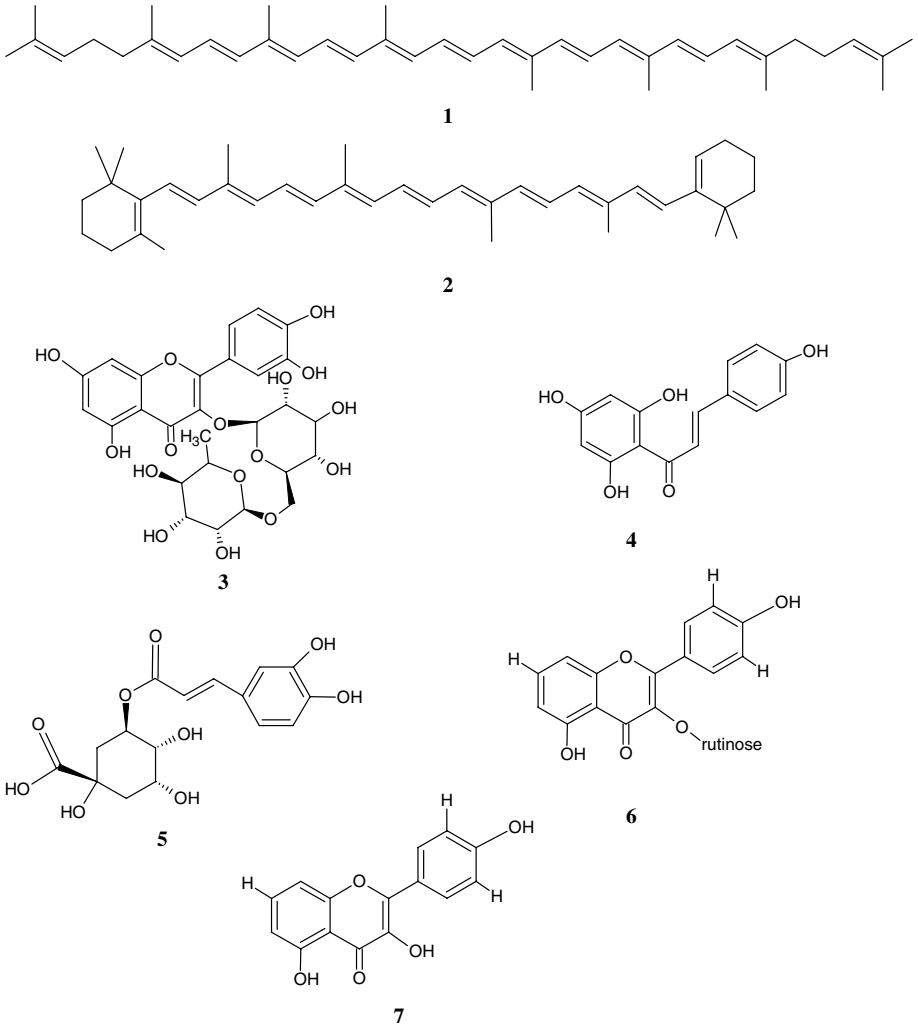

Fig. 1. Structures of compounds analysed in this study. **1**, lycopene; **2**, β-carotene; **3**, rutin; **4**, naringenin (chalcone); **5**, chlorogenic acid; **6**, kaempferol-3-*O*-rutinoside; **7**, kaempferol.

2000, 2002). More recently, tomato has become a focus for its phenolic antioxidant content, which has also been subject to manipulation (Schijlen et al., 2004). Both structural genes and transcription factors have been over-expressed leading to increased accumulation of flavonoids (Muir et al., 2001; Bovy et al., 2002). However, the extent of cross-talk between synthesis of antioxidant carotenoids and flavonoids, identified as consequences of transgenesis or mutation, is obviously important to understand at the genetic and metabolite level. This can be addressed by metabolic profiling of the fruit of lines altered in carotenoid or flavonoid pathways and initial data suggests limited influence of the relative pathways upon each other in tomato fruit (Long et al., 2006). With respect to manipulation of phenolic biosynthesis, however, there is a further consideration due to the branched nature of the pathway. The synthesis of the flavonoids, for example, branches from lignins following the 4-coumarate ligase step which occurs immediately after the cinnamate hydroxylation step. A major aspect of overall regulation appears to be at the level of transcription such that there is organ specificity in the expression of the respective pathways. Furthermore, consequences of manipulation of phenolic metabolism can give an insight into the relative roles of the various structural genes and the regulation of partitioning of carbon flux into the various components. However, it needs to be established whether perturbation of either pathway allows potential for redistribution of phenolic precursors by transport.

In addition to the manipulation of antioxidant flavonoids (Schijlen et al., 2004), there have now been a large number of studies on modifying lignin content and monomer composition as a means of potentially improving the efficiency of pulp and paper making for quality, economic and environmental reasons (Anterola and Lewis, 2002; Boudet et al., 2003). Thus most of the structural genes involved in lignin formation have now been downregulated in a number of plants including tobacco, Arabidopsis and poplar and the consequences on lignin amount and composition determined. Tobacco has been a model plant in this

endeavour due to its woody nature, ease of transformation and rapid generation of data compared with tree species.

Cinnamate 4-hydroxylase, which is a cytochrome P450 and a major flux control for lignification (Anterola and Lewis, 2002) has been found to exist in two distinct families, class I and class II, which are functionally identical but show around 60% sequence similarity. Some species such as Arabidopsis have class I only (Costa et al., 2003) while others, such as Solanaceous species, have both (Ehlting et al., 2006). Both forms have been subjected to sense and antisense manipulation in tobacco where class I (Sewalt et al., 1997) and class II forms of the enzyme (Blee et al., 2001) have been targeted with consequent lowering of lignin content. Although this data already exists in a Solanaceous species, tomato therefore poses an additional opportunity to study relative flux into lignin and dietary phenolics in fruit. This has already been addressed in a study, which utilized RNAi downregulation of lignin biosynthesis to enhance the availability of phenolic precursors and stimulate production of health-promoting compounds in the fruit (Van der Rest et al., 2006). A cinnamoyl CoA reductase was targeted which is specific to the lignification pathway. This led to downregulation of lignin and changes to the profile of the soluble phenolic pools of various organs with increased chlorogenic acid but without desired increases in fruit flavonoids. This suggests that partitioning of phenolic precursors is probably limited in redistribution amongst the organs.

We have attempted to increase antioxidant fruit flavonoids by utilising the potential flux control properties of C4H by overexpression since it has been repeatedly identified as a rate limiting step in the phenylpropanoid pathway (Anterola and Lewis, 2002). However, we show that sense manipulation of CYP73A24 leads to a consistent downregulation of stem lignin, probably as a result of sense suppression, but not fruit flavonoids. Although some lines showed increased flavonoid content together with a background of nearly normal levels of carotenoids and therefore potentially increasing the antioxidant content of the fruit, such advantages were offset by the developmental consequences as a result of alterations in lignin content.

2. Results

2.1. Generation of tomato plants engineered for sense expression of CYP73A24

The EST contig of 1708bp for CYP73A24 was used to design primers 5′- GAGCTCATGGATCTTCTCTTGCTGGAG-3′ (100FSac1) and 5′-TCTAGATTAGAAAGATCTTGGTTTCATC-3′ (101RXba1). These were used to amplify cDNAs reverse transcribed from total RNA extracted from ripe tomato fruit using an oligo $d(T)_{18}$. The identity of the resulting clones were confirmed by sequencing and the sequence showed closest similarity to other full length CYP73s from the Solanaceous plants, *Capsicum annum*, *C. chinense* and *Solanum tuberosum*. To assign which class of C4H CYP73A24 represents, the sequence was compared with tobacco, for which there are available full length sequences for the class II C4H protein from tobacco but only a partial sequence for the class I form (Fig. 2). CYP73A24 is clearly a class I C4H (Ehlting et al., 2006).

The complete tomato CYP73A24 (1515bp) coding region, amplified using the primers100FSac1 and 101RXba1 with a nos terminator ligated to the 5′ end, was cloned into the binary vector pBIS1N which had previously been digested with *Eco*R1 to remove the hmg1 insert and nos terminator. Ultimately the binary vector, renamed pBE:100F/101R, contained the coding region of tomato CYP73A24 in the sense orientation under the control of the CaMV35s promoter and nos terminator (Fig. 3a). The vector was transformed into tomato lines Moneymaker (*MMr*), a wild type line, and Old Gold Crimson (Og^c), a natural high pigment line. Initially 69 and 42 plants were confirmed positive by PCR in *MMr* and Og^c, respectively.

The effects of the C4H gene on plant phenotype were strikingly pleiotropic. In addition to plants with a normal appearance, four distinct other phenotypes were observed and classified as dwarf plants, plants with distorted (curly) leaves and pointed fruit, plants with long internodes and elongated growth and plants with thickened waxy leaves (Fig. 3b). In our experience, these phenotypes are unlikely to be consequences of the process of transgenesis alone as they were not observed with vector controls and they have not been observed before during transformation of tomato with a considerable number of carotenoid-related genes. Selected plants were grown to maturity. Some plants produced few flowers while others had abundant flowers but set few fruit. The range of flower types on the plants included those with large bulky dull green sepals such as those found on waxy plants e.g MMr5553 (Fig. 3b). The flower buds on some plants were very small and failed to open before they senesced and abscised.

Many single insert plants had no fruit. Waxy plants such as *MMr* 5553 had a small amount of fruit but often the base of the fruit had a blackened patch. The fruit comprising a truss were often small in size, with an occasional larger sized fruit. The small fruit usually contained no seed and the larger fruit yielded very small non-viable seed with the occasional larger seed. The curly leafed plants also tended to produce very little fruit, which again was often seedless. Fruit was often small and pointed while others had a flattened appearance. Seeds of Moneymaker transformed lines were often smaller than those of the control plants. Other phenotypic characteristics noted about the seeds included misshapen seeds and some which were very dark in colour. Nevertheless, it was possible to take through Moneymaker transformed plants containing a

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CYP73A24Ly      MD--LLLLEKTLIGLFFAI--------------------------LIAIIISKLRSK-R
CYP73SoTu       MD--LLLLEKTLIGLFFAI--------------------------LIAIIVSKLRSK-R
CYP73A21CaSi    ------------------------------------------------------------
CYP73CaAn       MD--LLLLEKTLVGLFFAI--------------------------VVAIIVSKLRSK-R
CYP73A27NiTa    MKNMAKLLNKTIFCILFTIAFLSFAKLLSSYLSMPFPLKYMSLIVPLLPLIINFLYVKPQ
CYP73A29NiTa    ---MAKLLNNTIFCILFSIVFLSFAKLLSSYLSIPFPLEYISLIVLLLPLIINFLCVKPQ


CYP73A24Ly      FKLPPGPIPVPIFGNWLQVGDDLNHRNLTEYAKKFGDVFLLRMGQRNLAVVSSPELAKEV
CYP73SoTu       FKLPPGPIPVPVFGNWLQVGDDLNHRNLTEYAKKFGDVFLLRMGQRNLVVVSSPELAKEV
CYP73A21CaSi    ------PIPVPVFGNWLQVGDDLNHRNLTDYAKKFGDIFLLRMGQRNLVVVSSPESAKEV
CYP73CaAN       FKLPPGPIPVPVFGNWLQVGDDLNHRNLTDYAKKFGDIFLLRMGQRNLVVVSSPESAKEV
CYP73A27NiTa    NNLPPGPTAVPIFGNWLQVGNDLNHQLLATMSQTYGPIFLLKLGSKNLAVVSNPELADQV
CYP73A29NiTa    NNLPPGPTAVPIFGNWLQVGNDLNHQLLATMSQTYGPIFLLKLGSKNLAVVSNPELANQV
                      * .**:********:****: *:  ::.:* :***::*.:**.***.** *.:*


CYP73A24Ly      LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRGGW
CYP73SoTu       LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRGGW
CYP73A21CaSi    LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRGGW
CYP73CaAN       LHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRGGW
CYP73A27NiTa    LHTQGVEFGSRPRNVVFDIFTGNGQDMVFTIYGDHWRKMRRIMTLPFFTNKVVHQYSDMW
CYP73A29NiTa    LHTQGVEFGSRPRNVVFDIFTGNGQDMVFTIYGDHWRKMRRIMTLPFFTNKVVHQYSDMW
                ***********.**********:*******:**:**********:********:** . *


CYP73A24Ly      ESEAASVVEDVKKNPESATNGIVLRKRLQLMMYNNMFRIMFDRRFESEDDPLFVKLKALN
CYP73SoTu       ESEAASVVEDVKKNPESATNGIVLRKRLQLMMYNNMFRIMFDRRFESEDDPLFVKLRALN
CYP73A21CaSi    ESEVASVVEDVKKNPESATNGIFLRKRLQLMMYNNMFRIMFDRRFESEDDPLFVKLRALN
CYP73CaAN       ESEVASVVEDVKKNPESATNGIVLRKRLQLMMYNNMFRIMFDRRFESEDDPPFVKLRALN
CYP73A27NiTa    ENEMDLVVNDLKKNEKVKYEGIVIRKRLQLMLYNIMYRMMFDAKFESQNDPLFIEATKFN
CYP73A29NiTa    ENEMDLVVDDLKKNEKVKYDGIVIRKRLQLMLYNIMYRMMFDAKFESQDDPLFIEATKFN
                *.*   **:*:*** :   :**.:*******:** *:*:*** :***::** *::   :*


CYP73A24Ly      GERSRLAQSFEYNYGDFIPILRPFLRGYLKICKEVKEKRLKLFKDYFVDERKKLANTKSM
CYP73SoTu       GERSRLAQSFEYNYGDFIPILRPFLRGYLKICKEVKEKRLKLFKDYFVDERKKLANTKSM
CYP73A21CaSi    GERSRLAQSFEYNYGDFIPIFRPFLRGYLKICKEVKEKRLQLFKDYFVDERKKLANTKSM
CYP73CaAN       AERSRLAQSFEYNYGDFIPILRPFLRGYLKICKEVKEKRLQLFKDYFVDERKKLSNTKSM
CYP73A27NiTa    SERSRLAQSFDYNYGDFIPLLRPFLRGYLNKCKDLQTRRLAFFNNYFVEKRRKIMD-ENG
CYP73A29NiTa    SERSRLAQSFDYNYGDFIPLLRPFLKGYLNKCKDLQTRRLAFFNNYFVGKRRKIMG-ENG
                .*********:********:*****:***: **::: :** :*::*** :*:*: . :.


CYP73A24Ly      DSNALKCAIDHILDAQQKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPHIQK
CYP73SoTu       DSNALKCAIDHILEAQQKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPHIQK
CYP73A21CaSi    DSNALTCAIHHILDAQQKGEINEDNVLYIVENINVAAIQTTLWSIEWGIAELVNHPHIQQ
CYP73CaAN       DSNALKCAIDHILEAQQKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPHIQQ
CYP73A27NiTa    EKHKISCAIDHIIDAEMKGEINEQNVLYIVENINVAAIETTLWSMEWAIAELVNHPIVQQ
CYP73A29NiTa    EKHKICCAIDHIIDAEMKGEISEQNVLYIVENINVAAIETTLWSMEWAIAELVNHPIVQQ
                :.: : ***.**::*: ****.*:***************:*****:**.******** :*:


CYP73A24Ly      KLRDEIDTVLGPGVQVSEPDMPKLPYLQAVIKETLRLRMAIPLLVPHMNLHDAKLAAYDI
CYP73SoTu       KLRDEIDTVLGPGMQVTEPDMPKLPYLQAVIKETLRLRMAIPLLVPHMNLHDAKLAGYDI
CYP73A21CaSi    KLREEIDTVLGPGVQVTEPDTQKLPYLQAVIKETLRLRMAIPLLVPHMSLHDAKLAVYDI
CYP73CaAN       KLREEIDAVLGPGVQVTEPDTHKLPDLQAVIKETLRLRMATPLLVPHMNIHDAKLAGYDI
CYP73A27NiTa    KIRDEISTVLK-GRSVTESNLHELPYLLATVNETLRLHTPIPLLVPHMNLEEAKLGGYTI
CYP73A29NiTa    KIRDEISTVLK-GKSVKESNLHELPYLLATVNETLRLHTPIPLLVPHMNLEEAKLGGYTI
                *:*:**.:**  * .*.*.:  :** * *.::*****: . *******.:.:***. * *


CYP73A24Ly      PAESKILVNAWWLANNPAHWKKPEEFRPERFLEEEKHVEANGN----DFRFLPFGVGSRS
CYP73SoTu       PAESKILVNAWWLANNPAHWKKPEEFRPERFFEEEKHVEANGN----DFRFLPFGVGRRS
CYP73A21CaSi    PAESKILVNAWWLANNPAHWKKPEEFRPERFLEEEKHVDANGN----DFRFLPFGVGRRS
CYP73CaAN       PAESKILVNPWWLANNPAHWKKPEEFRPERFLKEEKHVDANGN----DFRFLPFGVGRRS
CYP73A27NiTa    PKETKVVVNAWWLANNPAWWKNPNEFRPERFLEEDSSTEAAVAGGKVDFRYLPFGMGRRS
CYP73A29NiTa    PKETKVVVNAWWLANNPAWWKNQNEFRPERFLEEDSSTEAAVAGGKVDFRYLPFGMGRRS
                * *:*::**.******** **: :*******::*:. .:*       ***:****:* **


CYP73A24Ly      CPGIILALPILGITIGRLVQNFEMLPPPGQSKLDTSEKGGQFSLHILKHSTIVMKPRSF
CYP73SoTu       CPGIILALPILGITLGRLVQNFEMLPPPGQSKLDTSEKGGQFSLHILKHSTIVMKPRSF
CYP73A21CaSi    CPGIILALPILGITLGRLVQNFEMFPPPGQSKLDTSEKGGQFSLHILKHSTIVMKPRSF
CYP73CaAN       CPGIILALPILGITLGRLVQNFELLPPPGQSKLDTTEKGGQFSLHILKHSTIVMKPRSF
CYP73A27NiTa    CPGIILALPILGLVIAKLVSNFEMQGPPGVEKVDTSERGGQFSLHIAKHSTVVFKPIAA
CYP73A29NiTa    CPGIILALPILGLVIAKLVSNFEMQAPPGVGKVDTSEKGGQFSLHIAKHSTVVFKPIAA
                ***********:.:.:**.***:  ***  *:**:*:******** ****:*:** :
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Fig. 2. Comparative alignment of CYP73A24 against class I and class II C4H proteins from selected Solanaceous plants. Class I forms – *Solanum tuberosum* (CYP73SoTu; ABC6904), *Capsicum sinensis* (CYP73CaSi; AAC35857), *Capsicum annum* (CYP73CaAn; AAG43824) and two class II forms from tobacco (CYP73A27 and CYP73A29) are aligned and shows that CYP73A24 is a class I C4H.

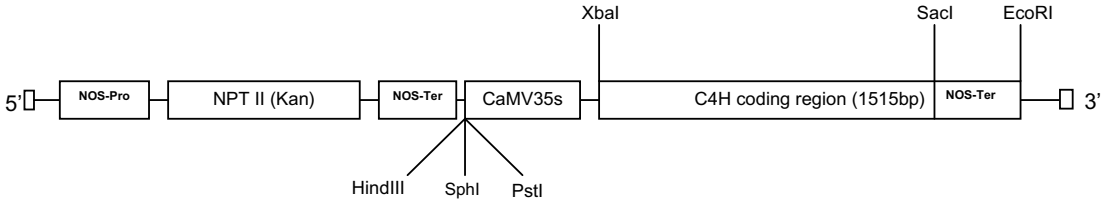

Fig. 3a. Schematic representation of the gene construct introduced into the Moneymaker and Old Gold Crimson tomato plant lines. The construct contained the coding sequence of tomato cinnamate 4-hydroxylase in the sense orientation under the control of a CamMV35s promoter and a Nos terminator.

Fig. 3b. Photographs of the four major phenotypes observed in tomato plants transformed with cinnamate 4-hydroxylase in the sense direction. Clockwise from top left: *MMr* 5652 (curly leaf), O^{gc}5482 (dwarf) *MMr* 5665 (waxy), *MMr*5467 (long internode).

single insert to the T_1 generation for metabolite and lignin analysis. No seed from T_0 Og^c fruit germinated. Therefore, all subsequent analysis was performed on lines transformed into Moneymaker (MMr) background.

2.2. Metabolomic analysis of transgenic tomato plants

Fruit, leaves and stems were analysed for phenolics and the profiles of the major compounds accumulated

determined. Complementary analysis of carotenoids was also performed to test for pleiotropic effects on their level due to possible cross-talk between the pathways. Flavonoid analysis was conducted on the fruit of all T_0 transformants, which produced fruit. (Fig. 4a). Changes in phenolic/flavonoid contents were observed but were not predictable, although some lines (5471, 5474, 5479 and 5482) showed desirable increases in rutin (**3**) and naringenin (**4**) (Fig. 4a). Three lines with single inserts showing the greatest changes in levels of flavonoids/phenolics were taken to the T_1 generation and homozygous lines identified. However, these did not produce fruit and further analysis of the effect of downregulating CYP73A24 was not possible. This lack of viability combined with the phenotypic analysis suggests importance of cinnamate 4-hydroxylase in development that was hitherto unappreciated. In comparison, possibly due to these developmental effects, the carotenoid levels were slightly lowered compared with wild type and the lines showed variable distribution between phytoene and lycopene (Fig. 5). The major leaf phenolics in the same lines were chlorogenic acid (**5**) and rutin (**3**) (Fig. 4b). These accumulated to a much higher levels in all transformed lines tested. Increases in chlorogenic acid (**5**) and rutin (**3**) were also observed in vegetative organs in tomato plants downregulated in CCR (Van der Rest et al., 2006). Chlorogenic acid-accumulation appears to be a usual consequence of engineering phenylpropanoid metabolism in Solanaceous species (Schadle et al., 2003) whereas in Arabidopsis cinnamoyl esters accumulate e.g. Abdulrazzak et al., 2006.

Stem sections examined by phloroglucinol staining (Fig. 6) show a range of intensity with several lines obviously downregulated for lignin content. When the lignin content was analysed by the MicroKlason method (Whiting et al., 1981) the wild type level was typical for that reported in a number of plants including tobacco with a content of around 20% (Table 1). However, unlike the phenolic content of the fruit of the transgenic lines, nearly all of them showed reduction of total lignin. These levels of reduction are greater than those seen in tobacco for heterologous antisense or partial sense expression of a Type I

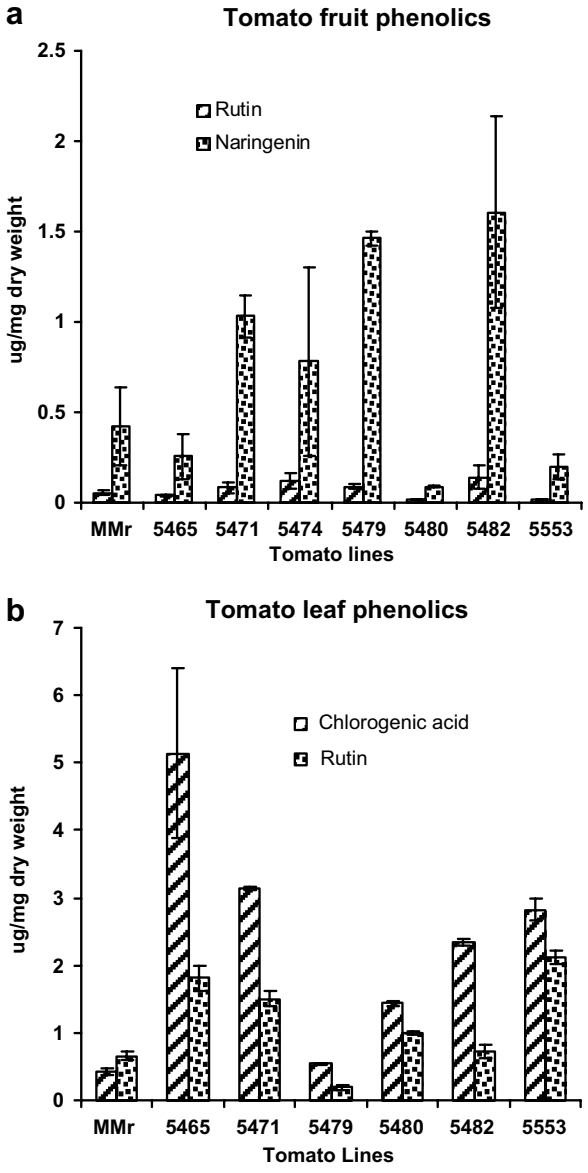

Fig. 4. (a) Phenolic content of fruit. Rutin and naringenin content of tomato fruit. Pericarp tissue including skin was typically taken from 3 tomatoes. The freeze–dried and powdered pericarp tissue was extracted with methanol containing salicylic acid as an internal standard. Three analytical replicates were performed. Data are expressed as means ± SE. (b) Phenolic profile of leaf. Leaf material was taken from three tomatoes and cut into strips and freeze dried. Tissue was extracted with methanol containing salicylic acid as an internal standard. Three analytical replicates were performed. Data are expressed as means ± SE.

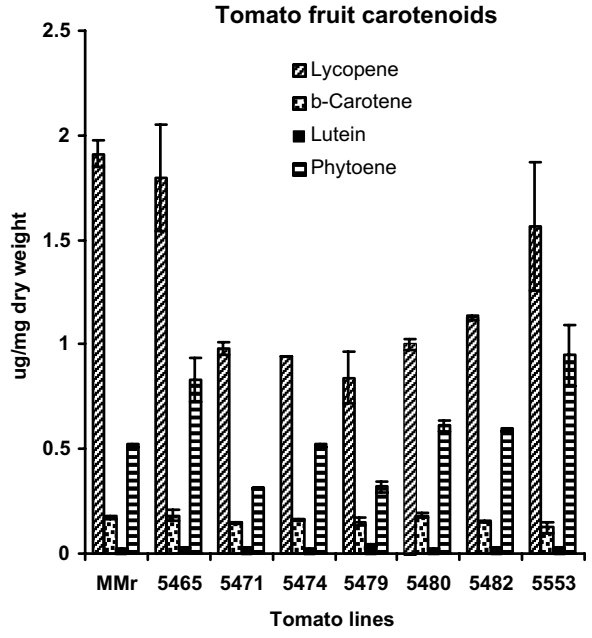

Fig. 5. Carotenoid content of fruit. Pericarp tissue including skin was typically taken from three tomatoes. The freeze–dried and powdered pericarp tissue was extracted with methanol and three analytical replicates were performed.

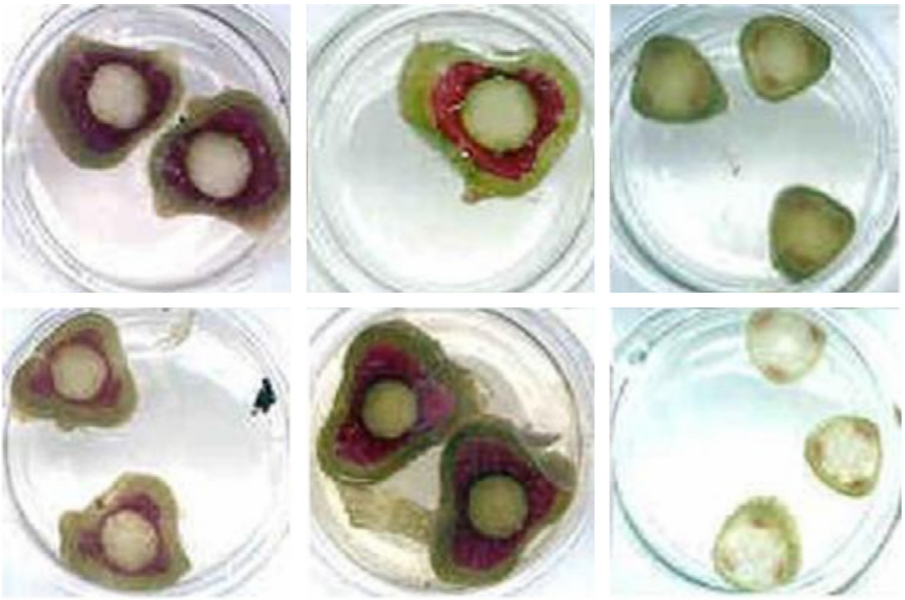

Fig. 6. Stem lignin staining. Phloroglucinol staining of basal sections of tomato stems. Clockwise from top left: *MMr* control, *MMr* 5480, *MMr* 5477 (dwarf), *MMr* 5539 (curly leaf), *MMr* 5553 (waxy), *MMr* 5468 (dwarf).

Table 1
Lignin content of stems

Lines	Lignin content (%)	Standard deviation	Phenotype
C 1	18.51	±1.27	Normal control
C 2	18.00	±1.06	Normal control
C 6-6	17.54	±1.45	Normal control
C 24-5	18.59	±3.69	Normal control
C 31-5	18.38	±1.59	Normal control
L 5543	13.84	±1.13	Normal
L 5465	15.82	±1.22	Normal
L 5466	12.50	±0.48	Normal
L 5470	7.56	±0.78	Normal
L 5471	16.08	±1.08	Normal
L 5474	13.25	±0.88	Normal
L 5476	17.24	±1.09	Normal
L 5479	11.18	±1.96	Normal
L 5480	19.74	±0.27	Normal
L 5481	8.99	±1.54	Normal
L 5482	7.06	±1.79	Normal
L 5646	16.16	±2.09	Normal
L 5468	9.35	±0.21	Dwarf
L 5477	10.73	±1.43	Dwarf
L 5675	18.86	±1.02	Dwarf
L 5539	12.84	±0.84	Curly leaf
L 5541	17.24	±0.45	Curly leaf
L 5542	14.17	±0.56	Curly leaf
L 5467	16.92	±0.93	Long internode
L 5472	11.36	±1.16	Long internode
L 5483	9.08	±2.01	Long internode
L 5548	13.67	±1.31	Waxy
L 5553	15.82	±0.82	Waxy

(Sewalt et al., 1997) or Type II (Blee et al., 2001) cinnamate 4-hydroxylase. Thus the level of lignin reduction is amongst the highest seen in any species or for downregulation of any structural gene within the lignin pathway (Anterola and Lewis, 2002). In both cases the effect on syringyl (S) levels was greater than on guaiacyl (G) units leading to a decreased S/G ratio overall. Until recently there were no reports of a homologous cinnamate 4-hydroxylase being downregulated. However, in the context of engineering for increased forage digestibility, the expression of a *Medicago truncatula* class I C4H cDNA in antisense orientation in *Medicago sativa* which show 99% similarity for class I C4H, resulted in much more efficient reduction in lignin (Reddy et al., 2005). This resulted in a seven-fold reduction in S-units.

Comparable analysis of G and S levels in tomato failed to show such preferential effects and indeed S levels increased relative to G levels (Fig. 7). Total Klason lignin content is plotted against thioacidolysis analysis for the ratio of G and S units for two control lines C1 and C2 against lines 5675 (dwarf phenotype), 5539 (curly leaf), 5483 (long internodes) 5548 (waxy) and 5481/82 (normal phenotype). This relative increase in S over G residues is understandable in terms of flux control where limitation

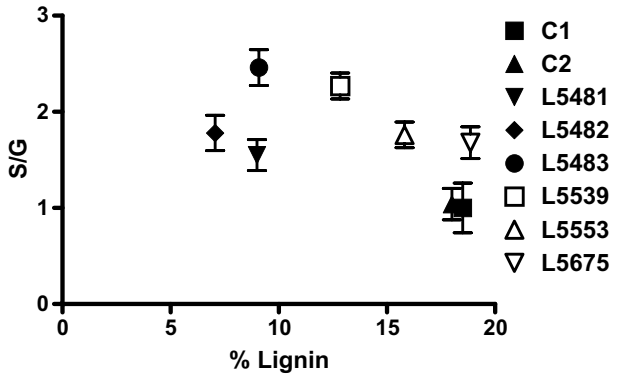

Fig. 7. Plot of G/S lignin against total lignin (see examples from Blee et al., 2001).

of the level of intermediates would preferentially accumulate end products if the pathway goes to completion.

2.3. Cyp73A24 expression in tomato lines

In order to understand the increase in flavonoids in some fruit lines, fruit from five tomato lines including Moneymaker control plants from the T_0 generation were selected for gene expression analysis using RT-PCR on the basis that they had single C4H gene inserts and showed a range of variation in fruit flavonoids compared to controls. Fig. 8a shows the results of this analysis. Expression levels of actin were used to normalise quantities of RNA used in the reactions. While variations in the level of PAL and C4H expression were observed, levels of CHS and FLS transcripts exclusively involved in flavonoid metabolism were identical. Plant lines 5465, 5480 and 5482 show clear overexpression of C4H compared to the Moneymaker control as expected. The levels of phenylalanine ammonia lyase (PAL) expression are likewise increased in these plants. Line 5474 shows wild-type expression for both. This does not, however, lead directly to increased accumulation of naringenin (**4**) since two of the overexpressors, 5465 and 5480 accumulate less in fruit.

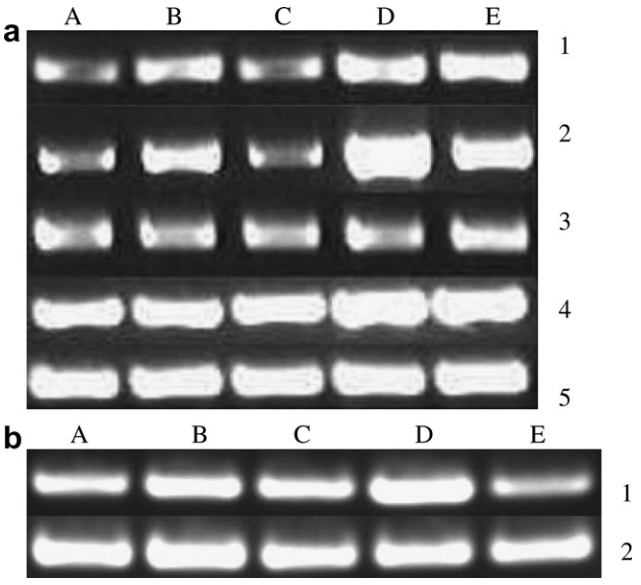

Fig. 8. (a) RT-PCR analysis of genes expressed as part of the phenylpropanoid pathway and flavonoid branch pathway. RT-PCR was performed on RNA isolated from 7 dpb fruit (three fruits per sample). Amplification was for 30 cycles for PAL, FLS actin and 35 cycles for C4H and CHS. Actin was used as reaction and gel loading control. Tomato lines were selected because of variations in flavonol and/or carotenoid content of fruit compared to controls. Column (A) *MMr* control; (B) *MMr* 5465; (C) *MMr* 5474; (D) *MMr* 5480; (E) *MMr* 5482. Row (1) PAL; (2) C4H; (3) CHS; (4) FLS; (5) actin. (b) RT-PCR analysis of C4H expression in tomato stems. RT-PCR was performed on stem sections from the tenth internode of mature tomato plants (three stems sections per sample). Amplification was for 30 cycles for C4H and actin. Actin was used as reaction and gel loading control. Column (A) *MMr* control; (B) *MMr* 5465; (C) *MMr* 5474; (D) *MMr* 5480; (E) *MMr* 5482. Row (1) C4H; (2) actin.

However, as in all lines tested it does lead to accumulation of chlorogenic acid (**5**) and rutin (**3**) in leaves to much higher levels than in wild type. This suggests mechanisms other than simple increases in gene expression account for up to four-fold increase in naringenin (**4**) seen in the fruit of some lines.

Stems were also analysed for *cyp73A24* expression (Fig. 8b). As for the fruit and leaves, the lines chosen had a range of lignin content with two lines displaying WT levels (5465 and 5480) with 5474 showing significant reduction and 5482 having a 61% reduction, amongst the lowest ever seen for an engineered plant. The latter two lines showed apparent partial silencing commensurate with the role of C4H being the major flux determinant for lignin biosynthesis (Anterola and Lewis, 2002).

3. Discussion

The present study was undertaken to determine the potential effect of the increase of flux into fruit phenolics and the consequent pleiotropic effects on metabolites and the determination of other unintended effects of transgenesis. Since the second enzyme of the pathway, cinnamate 4-hydroxylase, seems to be a major flux determinant (Anterola and Lewis, 2002) and is encoded by a single gene or small gene family it was chosen for an attempt to increase the antioxidant flavonoid content of fruit by sense overexpression. An inducible fruit-specific promoter such as those derived from the polyglacturonase (PG) and ACO gene was not used since CYP73A24 was expressed at low level in the fruit and they are inadequate for antisense or sense suppression with such genes. A CYP73A24 expressed in fruit was cloned and transformants produced for analysis of phenotype, flavonoids and carotenoids in the fruit, and lignin in the stem. Distinct developmental phenotypes arose and although they showed an inconsistent effect on fruit phenolics, they all showed reductions in stem lignin. Significant pleiotropic effects on fruit carotenoids were not shown as a consequence of downregulating carbon flux into phenolics.

The phenylpropanoids and flavonoids are potent antioxidants when tested in vitro (Rice Evans et al., 1995, 1997). Based on these findings, it was postulated that flavonoids may offer protection against major diseases such as coronary heart disease and cancer (Hertog and Hollman, 1996). The flavonoids, in particular, have been targets for enhancement in crops (Parr and Bolwell, 2000; Schijlen et al., 2004). 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. With respect to flavonoids, tomato contains rutin (**3**) (quercetin-3-rutinoside), kaempferol 3-*O*-rutinoside (**6**) and naringenin chalcone (**4**) (Crozier et al., 1997; Muir et al., 2001). Two strategies have proved successful in enhancing the level of these. In the first, chalcone isomerase from petunia

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 kaempferol (**7**) and naringenin (**4**) 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, caffeate, ferulate, sinapate and chlorogenic acid. Levels of these compounds have also been manipulated in programmes aimed at lowering monolignol intermediates and as a consequence lignin in cell walls (Boudet et al., 2003). Because the CYP73A24 cloned was expressed in ripening fruit, sense expression might be expected to increase the accumulation of fruit phenylpropanoids and flavonoids. As already shown for a number of mutants and lines transgenic for carotenoid and phenolic biosynthesis, it is possible to raise both types of antioxidants in fruit and there is little evidence for reciprocity when either pathway is upregulated (Long et al., 2006). This is true in the lines that showed increased flavonoid content with nearly normal carotenoid content produced in this study.

A large number of orthologues have now been described for C4H (CYP73A1-CYP73A51) allowing a detailed analysis of phylogeny (Ehlting et al., 2006) and individual species have either one or two genes. Arabidopsis, for example, appears to have one form of the enzyme (Costa et al., 2003). Some of the divergent members show about 60% sequence similarity to the initially cloned cinnamate 4-hydroxylases enabling them to be classified within the same family but they are sufficiently different to be classified as class II. CYP73A15 from French bean falls into this category and was shown to code for a cinnamate 4-hydroxylase following heterologous expression in yeast (Nedelkina et al., 1999). It has not been established hitherto why there is more than one form of the enzyme. So far CYP73A24, a class I C4H, is the only described full-length cinnamate 4-hydroxylase sequence from tomato. Inspection of the tomato unigene sequence indicates that the CYP73A24 (SGN-U143809; http://www.sgn.cornell,edu/search/uigene) is the predominant C4H sequence and shows expression in leaf, fruit pericarp and stem. Therefore, it can be deduced that this is the target of the sense expression since the other putative C4H (SGN-U143898) is highly restricted to flower, ovary and developing buds in its tissue specificity. Those species with two forms may have evolved these to respond to differing developmental and environmental stimuli or it could reflect subtle differences in specificity as has been found for the other cytochrome P450s involved in phenylpropanoid and lignin metabolism (Humphreys et al., 1999; Osakabe et al., 1999; Schoch et al., 2001).

In many lines in this study, over-expressing CYP73A24 resulted in considerable reduction of lignification which in some cases the lowest seen in any attempt to engineer lignin (Anterola and Lewis, 2002). Comparatively in tobacco, downregulation of the class I form CYP73A3 from Medicago, initially cloned as a stress response cDNA, in tobacco resulted in reduced Klason lignin contents and a decreased syringyl/guaiacyl ratio (Sewalt et al., 1997). The possible function of a class II enzyme has been investigated in transgenic tobacco by subjecting it to antisense and sense suppression using CYP73A15 from Phaseolus vulgaris (Blee et al., 2001). Lignin analysis gave comparable reductions in lignin to that found for CYP73A3. Preliminary evidence from model systems suggested that the class II C4H might also be related to vascular differentiation rather than stress responses and located to the Golgi apparatus (Smith et al., 1994; Nedelkina et al., 1999).

Analysing the accumulated data for transformations (Anterola and Lewis, 2002), it would appear that manipulation of targets in the phenylpropanoid pathway, the earliest stage of lignin biosynthesis, (PAL, C4H, 4CL and C3′H) in tobacco and Arabidopsis reduces the lignin content and, with the exception of PAL, generally shows higher G to S ratios (Kajita et al., 1996; Sewalt et al., 1997; Blee et al., 2001; Abdulrazzak et al., 2006). This may indicate that reduction of flux through the pathway leads to selective depletion of the intermediates that go through to S units and G units, since there is growing evidence that these pathways may be differentially regulated in different cell types. However in the present study there was an increase in S over G units as found for PAL in tobacco so there may be variation between species. In contrast, downregulation of the later enzymes of monolignol biosynthesis (coniferylaldehyde 5-hydroxylase (F5H), CCR and CAD) leads to limited effects on total lignin but with drastic and opposing changes in S/G ratios (Anterola and Lewis, 2002).

We have presented some evidence for partial silencing in the stems of lignin downregulated plants. There is a precedent for such co-suppression as a result of overexpression of a cytochrome P450 involved in phenylpropanoid metabolism leading to reductions in lignin content (Abdulrazzak et al., 2006; Ehlting et al., 2006). *Cyp98* codes for the coumaroyl-ester-3-hydroxylase (C3′H), the next hydroxylation step in the pathway after cinnamate 4-hydroxylation. Both null mutants and about 10% of overexpressing lines in Arabidopsis result in reduced lignin content, which was 63% of control in the latter case. In contrast, the co-suppressed lines accumulated three fold more flavonol glucosides from leaves than the corresponding controls and also produced purple anthocyanins (Abdulrazzak et al., 2006). Combined with significant developmental effects, the consequences of

overexpression on the phenotype is equally profound as we have found for the previous hydroxylase in the pathway in tomato. To further confirm the sometime unpredictable effects of manipulating cytochromes P450 in plants, antisense expression of CYP84A2 in tomato designed to elevate the antioxidant level of ferulate in fruit led to a four-fold increase in levels of rutin (**3**) instead (Rees, 2000; Long et al., 2006). The plants also showed accumulation of purple anthocyanins in the leaves (Rees, 2000) similar to that found for the co-suppressed CYP98A3 Arabidopsis lines (Abdulrazzak et al., 2006).

It is therefore important to understand why this level of downregulation is sufficient to affect the lignin pathway but not the accumulation of flavonoids to the same extent. It suggests there are subtle differences in the regulation of these pathways in stem and fruit. In the case of C3′H co-suppression, the ability to over accumulate flavonoids was ascribed to a non-redundant alternative *meta*-hydroxylation pathway while in this case there is the possible involvement of a second predicted C4H in tomato. Another contribution to such differential effects could be that overexpression is tolerated in fruit while local silencing occurs in tissues with high natural expression of the gene such as lignifying stem. Although the differential effect on lignin and fruit flavonoids is variable and unpredictable one consistent response of perturbation in Solanaceous species is the shunting of intermediates into chlorogenic acid (Schadle et al., 2003; Van der Rest et al., 2006). Such consistency is a good starting point for future exploration of the complex developmental regulation of phenolic metabolites.

4. Experimental

4.1. Materials

Standards for metabolic profiling were as described in Long et al., 2006.

4.2. Growth of tomato lines

Tomato plants were grown in a greenhouse with supplementary lighting. 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. Pericarp tissue including skin was typically from three tomatoes, freeze–dried and stored at −70 °C.

4.3. Generation of tomato plants transgenic for CYP73A24

Primers for cinnamate-4-hydroxylase were based on sequences from the Cytochrome P450 – CYP73A24 and tomato EST database TIGR tomato gene index – TC72100.

Total RNA isolated from ripe tomato fruit was reverse transcribed using an oligod(T) primer. Acquisition of full length cDNA clones for tomato C4H were obtained by amplification of this cDNA using the primers 5′- GAGCTCATGGATCTTCTCTTGCTGGAG-3′ (100FSac1) and 5′-TCTAGATTAGAAAGATCTTGGTTTCATC-3′ (101RXba1).

The binary vector pBIS1N was used for all the experiments. Within the T-DNA region, the pBIS1N constructs contained kanamycin resistance and, in the sense orientation with respect to the promoter, the entire full length cDNA. CaMV/35S promoter and nopaline synthase terminator were then used to regulate expression from the sense construct within the T-DNA. After transformation of *Agrobacterium tumefaciens* with pBIS1N antisense constructs, the cultures were initiated in 100 ml of Luria–Bertani medium containing 50 μg ml^{-1} kanamycin and allowed to grow for 12 h at room temperature with shaking. *A. tumefaciens* cells were collected by centrifugation and resuspended in liquid MS medium. Leaf strips were cut from genetically identical tomato plants (*L. esculentum* cv Moneymaker or *Og*^{c}), generated by vegetative propagation, then incubated in the resuspended *A. tumefaciens* cells for 20 min before being placed onto solid MS medium containing 1 mg ml^{-1} BAP and 0.1 mg ml^{-1} NAA for two days. The leaf strips were transferred to solid MS medium containing 1 mg ml^{-1} BAP, 0.1 mg ml^{-1} NAA, 500 mg ml^{-1} carbenicillin, and 100 mg ml^{-1} kanamycin and left for 5 weeks to form shoots. Shoots were excised from the leaf strips and inserted into solid MS medium containing 500 mg ml^{-1} carbenicillin and 100 mg ml^{-1} kanamycin and allowed 4 weeks for rooting. Regenerated plants were respectively screened for the presence of the transgene using PCR. DNA was isolated from leaf tissue using the Fast DNA Kit for plants (Bio 101, Carlsbad, CA, USA) and added to Ready to Go PCR beads (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) with CaMV/35S promoter and nopaline synthase terminator primers 5′-CAATCCCACTATCCTTCGC-3′ and 5′-CATCGCAAGACCGGCAACAG-3′, respectively. Cycling proceeded after 7 min at 95 °C with 40 cycles of 1 min at 95 °C, 1 min at 58 °C, and 2 min at 72 °C. Rooting on selective medium (described above) was repeated before transferring rooted plants to solid growth matrix and glasshouse grown exactly as described.

4.4. Analysis of transgenic plants

DNA was isolated from young leaf tissue using the DNeasy Plant Minikit (Qiagen, Crawley, West Sussex, UK). PCR was used to confirm the presence of the sense C4H construct using two pairs of primers. CAM35sF (5′-CAATCCCACTATCCTTCGC-3′) located in the CaMV/35S promoter and CYP73XF (5′-TTACGGTTTATGGTGAGCATTGG-3′) located in the CYP73A24 sense coding region, and NPTF (5′-GGCGTTCCTTGCGCAGCT-3′) and NPTR (5′-CGTGCTCGCTCGATGCGA-3′) both of which were located in the nopaline synthase gene. Cycling

proceeded after 2 min at 94 °C with 30 cycles of 40 s at 94 °C, 40 s at 55 °C, and 40 s at 72 °C. This was followed by a final step of 7 min at 70 °C.

4.5. Expression analysis

Total RNA (2 μg) was annealed to an anchored oligo dT(VN) (TTTTTTTTTTTTTTTVN) primer (1.1 μM) at 70 °C for 10 min and then immediately placed on ice for 10 min. Reverse transcription was in reverse transcription buffer (Promega) containing 0.5 mM dNTPs, and 200 units M-MLV reverse transcriptase (Promega) at 42 °C for 1 h in a total volume of 25 μl. The final concentration of the PCR was: primers 0.25 μM, dNTPs 0.2 mM, $MgCl_2$ 1.5 mM, and 1.5 μl cDNA in NH4 reaction buffer (Bioline) together with 0.625 units of BIOTAQ DNA polymerase (Bioline) in a total volume of 25 μl. The reaction commenced at 94 °C for 2 min, followed by a cycling stage of 94 °C 40 s, 55 °C 40 s and 70 °C 40 s. The cycling reaction was completed with a final incubation at 70 °C for 7 min. Primer sequences used for the reactions were as follows: C4H – forward primer, TTACGGTTTATGGTGAGCATTGG, reverse primer CACAATGTTGTTTGCATTGCAG; PAL – forward primer CAAGGGCTGGTGTGAAAGC, reverse primer GTCCTTCCTTGGGCTGCAAC; CHS – forward primer CGATCTTAGCCATTGGAAC, reverse primer GTAACTGCAGTGATCTCAG; FLS – forward primer GCAAGGGTCCAAGCAATATC, reverse primer GAGGCCATGACATTCTTGTC; actin – forward primer GACATGGAGAAGATTTGGC, reverse primer ATGAATACCAGCAGCTTCC.

4.6. Phenolic profiling: Extraction, separation and identification of phenolics in fruit

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. After centrifuging, MeOH was removed from the extracts under N_2. After cooling on ice, MeOH–H_2O (1:1) was added. Extracts were filtered before HPLC analysis. HPLC analysis was carried out on a Dionex Gynkotek system and analysed using Chromeleon sofware. 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) MeOH–H_2O (1:49 v/v) containing 0.015% HCl and (B) MeCN. The gradient program was A:B (95:5) for 10 min, followed by a linear gradient A:B (1:1). 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 using the Waters Millennium software supplied.

4.7. Carotenoid profiling: Extraction, separation and identification of carotenoids in fruit

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 ground freeze–dried material (1–2 mg), MeOH (100 μl) was added, along with the internal 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 $CHCl_3$ (400 μl). The pooled $CHCl_3$ extracts were dried under a stream of N_2 or by centrifugal evaporation. Dried residues were stored under an atmosphere of N_2 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.

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 C30, 5 μm column (250 × 4.6 mm) coupled to a 20 × 4.6 mm C30 guard (YMC Inc., Wilmington, NC, USA) with mobile phases consisting of MeOH (A), H_2O/MeOH (1:4 v/v) containing 0.2% ammonium acetate (B) and *t*-methyl butyl ether (C) was also used. The gradient elution used with this column was A:B (95:5, v/v) isocratically for 12 min, a step to A:B:C

(80:5:15) at 12 min, followed by a linear gradient to A:B:C (30:5:65) 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 EtOH in *n*-hexane (0.5:99.5, v/v) 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.

4.8. Lignin detection

Using a razor blade, 3 freehand sections 2 to 6 mm in thickness were excised from both the top and bottom of 60 cm sections of growing tomato plant stems starting approximately 2 cm above soil level. Immediately after being excised, the tissue sections from respective plants were placed in water filled microtiter-plate-wells. The two sets of sections from each plant were then subjected to lignin staining using the phloroglucinol–HCl method. Sections were stained in 12 well plastic tissue culture plates with 0.1% phloroglucinol in 70% ethanol. After incubation (15 min), the solution was removed and replaced with 6 M aqueous HCl. The sections remained in this solution until photographs were taken after approximately 50–60 min.

4.9. Determination of lignin content

Whole stems were harvested from mature ripe-fruit wild-type and transgenic tomato plants frozen in liquid nitrogen and freeze–dried. Cell wall residue was prepared as described previously (Chabannes et al., 2001) and Klason lignin contents were determined using a micro-Klason techniques (Whiting et al., 1981). Two plants from each line were pooled for extraction of the wall extract and measurements were performed in triplicate for each combined residue.

4.10. Thioacidolysis

S and *G* ratios were determined by thioacidolysis (Lapierre et al., 1986): Samples (14–16 mg) were individually dispersed with thioacidolysis reagent (15 ml, 0.2 M BF_3-etherate in a 8.75:1 (v/v) dioxane:ethanethiol) in a 20 ml glass tube fitted with a Teflon-lined screwcap under nitrogen atmosphere. Thioacidolysis was performed by placing each sample in a heating block held at 100 °C for 4 h. After cooling the reaction tubes in ice-water, each reaction mixture was individually poured into CH_2Cl_2 (20 ml). Each reaction tube was then rinsed with water (3 × 5 ml) with the aqueous solutions added to the reaction mixtures. To this was added internal standard (tetracosane C_{24}, ca. 0.8 mg) following which, each aqueous phase was adjusted to pH 3–4 with 0.4 M $NaHCO_3$. Each organic phase was next separated by decantation, with the aqueous phases resulting extracted with CH_2Cl_2 (3 × 30 ml); the combined organic phases were dried (anhydr. Na_2SO_4) and evaporated to dryness.

Each thioacidolysis residue was dissolved in CH_2Cl_2 (2 ml), with an aliquot (20 μl) silylated at room temperature with N,O bis (trimethylsilyl) trifluoroacetamide (BSTFA, 100 μl) in pyridine (40 μl) in a reaction vial (200 μl). The resulting derivatives were applied to a HP 6890 Series GC System equipped with a HP-5 (crosslinked 5% phenyl methyl siloxane, 30 mm × 0.25 mm × 0.25 μm) column; product identity was established by reference to retention times of authentic silylated standards, and by mass spectrometric analyses using the HP 5973 MS detector (EI mode, 70 eV). Column conditions: 160–250 °C at 2 °C/min until the final temperature, this being held at 250 °C for a further 5 min. For quantitative analyses, authentic standards (G-CHSEt-CHSEt-CH_2SEt and S-CHSEt-CHSEt-CH_2SEt) were used to calculate the response factors for both monomeric *G* and *S* units, respectively. All samples were analyzed in duplicate.

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References

Abdulrazzak, N., Pollet, B., Ehlting, J., Larsen, K., Asnaghi, C., Ronseau, S., Proux, C., Erhardt, M., Seltzer, V., Renou, J-P., Ullmann, P., Pauly, M., Lapierre, C., Werck-Reichhart, D., 2006. A *coumaroyl-ester-3-hydroxylase* insertion mutant reveals the existence of non-redundant *meta*-hydroxylation pathways and essential roles for phenolic precursors in cell expansion and plant growth. Plant Physiology 140, 30–48.

Anterola, A.M., Lewis, N.G., 2002. Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry 61, 221–294.

Blee, K.A., Choi, J.W., O'Connell, A.P., Jupe, S.C., Schuch, W., Lewis, N.G., Bolwell, G.P., 2001. Antisense and sense expression of cDNA coding for CYP73A15, a class II cinnamate 4-hydroxylase, leads to a delayed and reduced production of lignin in tobacco. Phytochemistry 57, 1159–1166.

Boudet, A.-M., Kajita, S., Grima-Pettenati, J., Goffner, D., 2003. Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends in Plant Science 8, 576–581.

Bovy, A., de Vos, R., Kemper, M., Schijlen, E., Almenar Pertejo, M., Muir, S., Collins, G., Robinson, S., Verhoeyen, M., Hughes, S., Santos-Buelga, C., van Tunen, A., 2002. High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. Plant Cell. 14, 2509–2526.

Bramley, P.M., 2000. Is lycopene beneficial to human health? Phytochemistry 54, 233–236.

Bramley, P.M., 2002. Regulation of carotenoid formation during tomato fruit ripening and development. Journal of Experimental Botany 53, 2107–2113.

Chabannes, M., Barakate, A., Lapierre, C., Marita, J.M., Ralph, J., Pean, M., Danoun, S., Halpin, C., Grima-Petenatti, J., Boudet, A.-M., 2001. Strong decrease in lignin content without significant alteration of plant

development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. Plant Journal 28, 257–270.

Costa, M.A., Collins, R.E., Anterola, A.M., Cochrane, F.C., Davin, L.B., Lewis, N.G., 2003. An in silico assessment of gene function and organization of the phenylpropanoid pathway metabolic networks in *Arabidopsis thaliana* and limitations thereof. Phytochemistry 64, 1097–1112.

Crozier, A., Lean, M.E.J., McDonald, M.S., Black, C., 1997. Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuces, and celery. Journal of Agricultural and Food Chemistry 45, 590–595.

Ehlting, J., Hamberger, B., Million-Rousseau, R., Werck-Reichhart, D., 2006. Cytochromes P450 in phenolic metabolism. Phytochemistry Reviews 5, 239–270.

Fraser, P.D., Pinto, M.E., Holloway, D.E., Bramley, P.M., 2000. Application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. Plant Journal 24, 551–558.

Hertog, M.G.L., Hollman, P.C.H., 1996. Potential health effects of the dietary flavonol quercetin. European Journal of Clinical Nutrition 50, 63–71.

Humphreys, J.M., Hemm, M.R., Chapple, C., 1999. New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. Proceedings of the National Academy of Sciences of the United States of America 96, 10045–10050.

Kajita, S., Katayama, Y., Omori, S., 1996. Alterations in the biosynthesis of lignin in transgenic plants with chimeric genes for 4-coumarate: coenzyme A ligase. Plant and Cell Physiology 37, 957–965.

Lapierre, C., Monties, B., Rolando, C., 1986. Thioacidolysis of poplar lignin: identification of monomeric syringyl products and characterization of guaiacyl-syringyl lignin fraction. Holzforschung 40, 113–118.

Long, M., Millar, D.J., Kimura, Y., Donnovan, G., Rees, J., Fraser, P., Bramley, P.M., Bolwell, G.P., 2006. Metabolite profiling of carotenoid and phenolic pathways in mutant and transgenic lines of tomato: identification of a high antioxidant fruit line. Phytochemistry 67, 1750–1757.

Muir, S.R., Collins, G.J., Robinson, S., Hughes, S., Bovy, A., De Vos, R., van Tunen, A.J., Verhoeyen, M.E., 2001. Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. Nature Biotechnology 19, 470–474.

Nedelkina, S., Jupe, S.C., Blee, K.A., Schalk, M., Werck-Reichhart, D., Bolwell, G.P., 1999. Novel characteristics and regulation of a divergent cinnamate 4-hydroxylase (CYP73A15) from French bean: engineering expression in yeast. Plant Molecular Biology 39, 1079–1090.

Osakabe, K., Tsao, C.C., Li, L., Umezawa, T., Carraway, D.T., Smeltzer, R.H., Joshi, C.P., Chiang, V.L., 1999. Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. Proceedings of the National Academy of Sciences of the United States of America 96, 8955–8960.

Parr, A.J., Bolwell, G.P., 2000. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. Journal of the Science of Food and Agriculture 80, 985–1012.

Reddy, M.S.S., Chen, F., Shadle, G., Jackson, L., Aljoe, H., Dixon, R.A., 2005. Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.). Proceedings of the National Academy of Sciences of the United States of America 102, 16537–16578.

Rees, J., 2000. Engineering phenolic antioxidant properties in tomato fruit. Ph.D. Thesis, London University.

Rice Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M., Pridham, J.B., 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radical Research 22, 375–383.

Rice Evans, C.A., Miller, N.J., Paganga, G., 1997. Antioxidant properties of phenolic compounds: The polyphenolic content of fruit and vegetables and their antioxidant activities. What does a serving constitute? Trends in Plant Science 2, 152–159.

Schadle, G.L., Wesley, S.V., Korth, K.L., Chen, F., Lamb, C., Dixon, R.A., 2003. Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of L-phenylalanine ammonia-lyase. Phytochemistry 64, 153–161.

Schijlen, E.G.W.M., de Vos, C.H.R., van Tunen, A.J., Bovy, A.G., 2004. Modification of flavonoid biosynthesis in crop plants. Phytochemistry 65, 2631–2648.

Schoch, G., Goepfert, S., Morant, M., Hehn, A., Meyer, D., Ullmann, P., Werck-Reichhart, D., 2001. CYP98A3 from *Arabidopsis thaliana* is a 3′-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. Journal of Biological Chemistry 276, 36566–36574.

Sewalt, V.J.H., Ni, W., Blount, J.W., Jung, H.G., Masoud, S.A., Howles, P.A., Lamb, C.J., Dixon, R.A., 1997. Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. Plant Physiology 115, 41–50.

Smith, C.G., Rodgers, M.W., Zimmerlin, A., Ferdinando, D., Bolwell, G.P., 1994. Tissue and subcellular immunolocalisation of enzymes of lignin synthesis in differentiating and wounded hypocotyl tissue of French bean (*Phaseolus vulgaris* L.). Planta 192, 155–164.

Van der Rest, B., Danoun, S., Boudet, A-M., Rochange, S.F., 2006. Down-regulation of cinnamoyl-CoA reductase in tomato (*Solanum lycopersicum* L.) induces dramatic changes in soluble phenolic pools. Journal of Experimental Botany 57, 1399–1411.

Whiting, P., Favis, B.D., St-Germain, F.G.T., Goring, D.A.L., 1981. Fractional separation of middle lamella and secondary tissue from spruce wood. Journal of Wood Chemistry and Technology 1, 29–42.