Hydroxycinnamic acids and UV-B depletion: profiling and biosynthetic gene expression in flesh and peel of wild-type and *hp-1*

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***Summary**

Hydroxycinnamic acids (HCAs) are phenolic compounds widely found in most plant families. Aim of the present work was to investigate their accumulation and biosynthetic gene expression in presence or absence of UV-B radiation in tomato fruits of wild-type and *hp-1*, a mutant characterized by exaggerated photoresponsiveness and increased fruit pigmentation. Gene expression and HCAs content were higher in *hp-1* than in wild type peel and UV-B depletion determined a decrease in HCAs accumulation in wild-type and an increase in *hp-1* fruits, generally in accordance with biosynthetic gene expression. In flesh, despite a similar transcript level of most genes between the two genotypes, HCAs content was generally higher in wild type than in *hp-1*, although remaining at a lower level with respect to wild type peel. Under UV-B depletion, a general reduction of HCAs content was observed in wild-type flesh, whereas an increase in the content of p-coumaric acid and caffeic acid was observed in *hp-1* flesh.

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ABSTRACT

Hydroxycinnamic acids (HCAs) are phenolic compounds widely found in most plant families. Aim of the present work was to investigate their accumulation and biosynthetic gene expression in presence or absence of UV-B radiation in tomato fruits of wild-type and *hp-1*, a mutant characterized by exaggerated photoresponsiveness and increased fruit pigmentation. Gene expression and HCAs content were higher in *hp-1* than in wild type peel and UV-B depletion determined a decrease in HCAs accumulation in wild-type and an increase in *hp-1* fruits, generally in accordance with biosynthetic gene expression. In flesh, despite a similar transcript level of most genes between the two genotypes, HCAs content was generally higher in wild type than in *hp-1*, although remaining at a lower level with respect to wild type peel. Under UV-B depletion, a general reduction of HCAs content was observed in wild-type flesh, whereas an increase in the content of p-coumaric acid and caffeic acid was observed in *hp-1* flesh.

Keywords: Hydroxycinnamic acids; RT-PCR; Tomato; *Lycopersicon esculentum*; UV-B depletion.

INTRODUCTION

Hydroxycinnamic acids (HCAs) are secondary metabolites derived from phenylalanine (Fig. 1) and are widely distributed in plant species that are consumed as food or used for beverages, in particular they are present at high concentrations in fruits, vegetables, tea, cocoa and wine. They can be found as free carboxylic acid, amides or esters formed by condensation with hydroxylic acids, flavonoids or carbohydrates (Bate-Smith, 1956; Mattila and Hellström, 2007). HCAs are health protecting component in the human diet as a result of their free radical scavenging capacity, able to prevent DNA and lipid oxidation by reactive oxygen species (reviewed in (El-Seedi et al., 2012)). They are

potential therapeutic agents for neurodegenerative diseases, such as Alzheimer and Parkinson, and in prevention of cardiovascular disease and diabetes. They are also considered potential cancer inhibitors and protectors against side effects of chemotherapy (reviewed in (El-Seedi et al., 2012)). HCAs, in particular ferulic and p-coumaric acid, have been utilised as potent UV-radiation absorbent compounds used as ingredients in topical UV-shielding agents, such as sunscreens, to counteract skin damages due to reactive oxygen species produced by UV light exposure (Sander et al., 2004).

HCAs are produced by a phenylpropanoid metabolic network that gives rise to flavonoids, anthocyanins, lignins, lignans, stilbenes, chalcones and coumarins (Fig. 1). They are synthesized from phenylalanine, via its deamination by phenylalanine ammonia-lyase (PAL), followed by three steps of hydroxylation by cinnamate 4-hydroxylase (C4H), *p*-coumaroyl ester 3-hydroxylase (C3H) and ferulate 5-hydroxylase (F5H) that produce coumaric acid, caffeic acid and 5-hydroxy ferulic acid, respectively. The 3-*O*-methyltransferase (*COMT*) enzyme catalyses the production of ferulic and sinapic acid from caffeic acid and 5-hydroxy ferulic acid, respectively, whereas 4-coumarate:CoA ligase (*4CL*) catalyses the formation of HCA-CoA by using as substrate different HCAs (Wink, 2010).

Two *R2R3-MYB* genes have been shown to be negative regulators of the HCA biosynthesis. *AmMYB308* was isolated from *A. majus* and when overexpressed in tobacco caused an inhibition of hydroxycinnamic and monolignol biosynthesis (Tamagnone et al., 1998). *AtMYB4* from Arabidopsis, orthologous to *AmMYB308,* is a repressor of cinnamate 4-hydroxylase, thereby negatively modulating accumulation of sinapate esters sunscreens in leaves. In presence of UV-B light, *AtMYB4* is repressed, thus resulting in an increase of sinapate esters production in leaves (Jin et al., 2000).

Effect of UV-B radiation (280–320 nm) in plants vary with the fluence rate of exposure (Brosché and Strid, 2003; Frohnmeyer and Staiger, 2003). High fluence rates of UV-B can induce the formation of reactive oxygen species causing damage to DNA, proteins, lipids, membranes and

tissue necrosis (A-H-Mackerness et al., 2001; Frohnmeyer and Staiger, 2003), stimulating the expression of genes involved in the perception and signalling of stress, wound and defence responses (A-H-Mackerness et al., 2001; Stratmann, 2003). Natural fluence rates of UV-B, normally present in sunlight, promote metabolic, photomorphogenic and developmental changes, stimulating the synthesis of flavonoids which provide, together with other phenolic compounds, a UV-protecting sunscreen in epidermal tissues (Jin et al., 2000; Li et al., 1993; Winkel-Shirley, 2001). Molecular characterization of *hp-1* and *hp-2* tomato mutants, which exhibit exaggerated light responsiveness, revealed that *HP1* and *HP2* genes encode tomato homologues of the light signal transduction proteins DDB1a and DET1 from Arabidopsis, respectively. These proteins interact and participate to the formation of the CDD complex, together with COP10 (Schroeder et al., 2002; Yanagawa et al., 2004). CDD complex and COP1 were shown to interact with a scaffold protein CULLIN4 (CUL4) to form a heterogeneous group of E3 ligases that regulate multiple aspects of the light regulation (Chen et al., 2010). Recent studies demonstrated that in tomato, HP1/SlDDB1 and HP2/SlDET1 are essential components of CUL4-based E3 ligase complex, in which SlDDB1 is associated with tomato CUL4 and SlDET1 (Wang et al., 2008).

Previous studies indicated that UV-B radiation significantly affect the total phenolic content of fully ripe tomato fruits, so that the total concentration of caffeic acid, p-coumaric acid and ferulic acid in two different commercial cultivars was approximatively 20% lower in UV-B depleted fruits compared to fruits grown under ambient solar UV radiation (Luthria et al., 2006). In addition, we have previously shown that UV-B depletion enhanced HCAs content of a commercial line with low lycopene content (Esperanza), and decreased HCAs in a lycopene-rich line (DRW 5981), thus indicating that the effect of UV-B on HCAs accumulation in commercial cultivars is genotype-dependent (Giuntini et al., 2008). The present work was addressed to extend our knowledge on the molecular events underlying HCAs accumulation both in tomato flesh and peel by analysing wild-type and *hp-1* mutant fruits grown in the presence or absence of UV-B light. To this purpose, not

only the HCAs content, in particular of p-coumaric acid, caffeic acid, ferulic acid and sinapic acid, but also the expression of the main HCAs biosynthetic genes have been analysed during ripening.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of the *hp-1* tomato (*Lycopersicon esculentum*) mutant and the near isogenic corresponding wild-type cv. Money Maker, obtained from the Tomato Genetics Resource Center (http://tgrc.ucdavis.edu/), were sown in plug trays with a peat/perlite (3:1 v/v) medium. Seedlings were transplanted into pots containing a peat/pumice/commercial soil mixture (1:1:1 v/v) and cultivated within growth chamber at 17/25°C (night/day) and a relative humidity of 60–80% under a 14/10 h light/dark photoperiod, with a photon flux density at plant height of 530 μmol photons m^{-2} s^{-1}. UV-A and UV-B radiation accounted for 4.95% and 0.16% of total irradiance, respectively. About 45 days after sowing, when the seedlings had reached the stage of four true leaves, the tomato plants were again transplanted into bigger pots (26 cm diameter) containing the same medium and 2 g of L-1 of 28N-8P-16K controlled-release fertilizer and irrigated daily. Pots were placed randomly under two different tunnels to avoid the border effect. Tunnels were located in an open field, and their longest sides were directed along the west−east direction to allow for uniform exposure of plants to sunlight. The experiment was carried out in the spring and summer (3 May to 25 July) of 2010 at Pisa, Italy (43°43′N, 10°23′E). Mean irradiance measured at midday was 2037 ± 156 and 1988 ± 148 μmol photons m^{-2} s^{-1} under control and UV-B deprived tunnels, respectively. UV-A and UV-B radiation were 102.9 ± 7.5 and 7.8 ± 0.7 μmol photons m^{-2} s^{-1} under control tunnel and 75.8 ± 6.1 and 0.8 ± 0.1 μmol photons m^{-2} s^{-1} in UV-B deprived tunnel. The first tunnel was covered by polyethylene film transparent to the whole sunlight spectrum while the second

tunnel was covered by polyethylene film stabilized with the UV-B absorber benzophenone (Agraria Di Vita, Pistoia, Italy) as previously described (Calvenzani et al., 2010; Lazzeri et al., 2012).

Healthy fruits of comparable dimension were carefully harvested from sunny branches at the mature green (MG, 35−40 Days Post Anthesis), turning (TU, breaker +3), and red ripe (RR, breaker +7) stages, in accordance with the procedure reported by Grierson and Kader (1986). Fruits were harvested three times at 2-3 days intervals and each harvest represented a biological replicate constituted by 15 fruits collected from five plants. Samples of peel and flesh (fruit minus peel) collected from each bulk of 15 fruits were pooled together, frozen by liquid nitrogen, and stored at −80 °C until analysis.

Extraction and quantification of Hydroxycinnamic Acids.

Freeze-dried samples of tomato flesh and peel (1 g fresh weight) were extracted for 30 min under magnetic stirring with 20 mL of 80% methanol aqueous solution, BHT (0.1% w/w) to prevent oxidation and *o*-coumaroyl methyl ester as internal standard. The mixture was centrifuged at 14000 x g for 15 min at 4°C and the supernatant was recovered. The procedure was repeated two additional times, the extracts were combined and reduced to 15 mL by rotavapor.

Quantitative analysis of hydroxycinnamic acids (p-coumaric acid, caffeic acid, ferulic acid and sinapic acid) was performed after alkaline hydrolysis in order to obtain the free forms, as these compounds are preferentially present as glucose or quinic esters in many fruits. Alkaline hydrolysis was performed at 25 °C in the dark for 4 h, by adding 5 mL of 1 M NaOH to the supernatant (10 mL). After neutralization with 1 M HCl, the hydrolyzed fraction was extracted twice with ethyl acetate (10 mL), the organic solution was pooled and evaporated under vacuum, and the residue was dissolved in methanol (1 mL) and injected in HPLC (Rapisarda et al., 1998), after filtration through 0.45-µm filters (Sartorius Stedim Biotech, Goettingen, Germany).

Separation was performed by a Spectra System P4000 HPLC, equipped with a UV 6000 LP photodiode array detector (Thermo Fisher Scientific, Waltham, MA) using a Phenomenex Prodigy

LC-18 RP column (5 µm particle size, 250 x 4.6 mm, Phenomenex Italia, Castel Maggiore, Italy). The mobile phase flow rate was fixed at 1.0 mL/min. Hydroxycinnamic acids were eluted using H_2O, pH 2.7 with formic acid (solvent A) and methanol (solvent B), in the following proportion: 0-20 min, 100% A; 20-38 min, 60% A and 40% B; 38-45 min, 100% A. Identification and quantification were carried out using commercial standards of coumaric, ferulic, sinapic and caffeic acid (Sigma Aldrich Chemical Co, St. Louis, MO) at 287 (ferulic and caffeic acid) and 320 nm (coumaric and sinapic acid).

RNA Isolation and Gene Expression Analyses

RNA was isolated from samples of 3 g of flesh and peel from wild-type and *hp-1* fruits as previously described (Calvenzani et al., 2010). About 5 µg of total RNA was reverse-transcribed using the RT Superscript™ II (Invitrogen, Carlsbad, CA) and, after first strand cDNA synthesis using an oligo dT as previously described (Procissi et al., 1997), the samples were diluted 50 fold and used as templates for real time RT-PCR analysis of genes indicated in Table 1.

Quantitative real time RT-PCR analysis was performed using SYBR Green with the Cfx96™BioRad Real Time system in a final volume of 20 µL containing 5 µL of 50-fold diluted cDNA, 0.2−0.4 µM of each primer, and 10 µL of 2X SOS Fast EVA-Green Supermix (BioRad Laboratories, Hercules, CA). Oligonucleotides used as primers are indicated in Table 1. As a reference for normalization, we used the *LeEF1* gene, encoding the tomato ELONGATION FACTOR 1-α, because of its high and stable expression in mature tomato fruit (Bartley and Ishida, 2003) by using primers LeEF1-F4 and LeEF1-R3. Relative quantification was analyzed using Cfx Manager Software version 1.6 (BioRad Laboratories).

The protocol used was as follows: 95 °C for 2 min, 55 cycles of 95 °C for 15 s, and 60 °C for 30 s. A melt curve analysis was performed following every run to ensure a single amplified product for

each reaction. Relative quantification of the target RNA expression level was performed using the comparative Ct method (UserBulletin 2, ABI PRISM7700 Sequence Detection System, Dec 1997; Perkin- Elmer Applied Biosystems) in which the differences in the Ct (threshold cycle) for the target RNA and endogenous control RNA, called ΔCt, were calculated to normalize for the differences in the total amount of cDNA present in each reaction and the efficiency of the reverse transcription. Finally, the target RNA expression level was obtained from the equation $2^{-\Delta\Delta Ct}$ and expressed relative to a calibrator (wild-type flesh of fruits at MG stage grown under control conditions). Standard errors of Ct values were obtained from measurements performed in triplicate.

Statistical analysis

At any ripening stage, hydroxycinnamic acids and RNA were extracted from peel and flesh collected from three groups of fruits each consisting of 15 berries. Samples for hydroxycinnamic acids and RNA analyses were collected from the same plants. Values shown in the figures are means of three replicates ± SE.

Shapiro-Wilk and Anderson-Darling tests and Levene test were applied to check for normal distribution of data and for homogeneity of variance, respectively. Data were analysed separately for peel and flesh by three-way ANOVA to check for differences due to genotype, UV-B radiation, ripening stage and their interactions, followed by Tukey–Kramer *post hoc* test at the 0.05 significance level using NCSS 2000 (NCSS Statistical Software, Kaysville, Utah, USA) statistical software.

RESULTS AND DISCUSSION

With the aim of understanding how UV-B light can modulate the biosynthesis of HCAs in tomato fruits during ripening, we analysed the accumulation of HCAs and the expression of the corresponding biosynthetic genes in wild-type and *hp-1* fruits at three different ripening stages (MG mature green, TU turning, RR red ripe) obtained from plants grown under plastics designed to transmit full sunlight UV (control fruits) or no UV-B radiations (UV-B-depleted fruits). Moreover, peel and flesh have been analysed separately, in order to determine whether UV-B light differentially affected the HCAs accumulation and the gene expression in those tissues (Fig. 2 and 3).

HCAs content and gene expression in peel of wild type and *hp-1* in control conditions

Our analyses revealed that in peel of control fruits, the expression levels of most biosynthetic genes analysed were significantly higher in *hp-1* compared to wild type (compare Fig. 2a,b). Specifically, at TU stage *COMT* and *C3H* showed a transcript level 19- and 11-fold higher than in wild type respectively, whereas the transcript levels of *PAL*, *4CL* and *F5H* were 1.3-, 2- and 2.6-fold higher than in wild type, respectively.

In wild-type peel, some genes showed a significant peak of activation at TU stage (*PAL*, *C4H*, *4CL*, Fig. 2a) compared to MG stage, followed by a transcript decrease at RR stage (Fig. 2a), whereas *COMT* transcription was not detectable under control light conditions (Fig. 2a). In *hp-1* peel, the expression profile of all the analysed genes showed a significant peak of expression at TU stage compared to MG stage, including *COMT* that was expressed at very low levels at MG and TU stages (Fig. 2b).

In general, HCAs followed the same trend of gene expression. In fact, in wild-type peel HCAs were present during all fruit maturation stages, although at low concentrations at RR stage (Fig. 2c). In detail, p-coumaric acid showed a significant peak of accumulation at TU stage compared to MG

stage and a decrease at RR stage (Fig. 2c), according to the *PAL* and *C4H* expression profile (Fig. 2a). Also caffeic acid, the most abundant at MG and TU stage, with similar levels in both stages, significantly decreased at maturation (Fig. 2c), consistent with the transcript level of *C3H*, that was more expressed during MG and TU stages and then significantly decreased at RR stage (Fig. 2a). The undetectable expression level of *COMT*, which is responsible for conversion of caffeic acid to ferulic acid and of 5-hydroxy ferulic acid to sinapic acid, is consistent with the accumulation of caffeic acid and the very low accumulation of ferulic acid and sinapic acid (Fig. 2a,c), which may be in part synthesized with very low efficiency by the alternative route involving *4CL* (Fig. 1). A marked difference in COMT mRNA levels, protein levels and enzyme activity have been reported in two wheat cultivars and associated with stem strength and lignin synthesis from the precursors ferulic and sinapic acids. In particular, a lower expression level of the *COMT* gene was responsible for a reduced content of lignin in stems of a lodging-sensitive wheat cultivar (Ma, 2009).

In *hp-1* peel, according to the *PAL* and *C4H* expression profile (Fig. 2b), p-coumaric acid presented a significant peak at TU stage compared to MG stage and decreased at RR stage. Accumulation of caffeic acid and ferulic acid was anticipated with respect to wild type, as they were detected at MG stage at significant higher level than in wild type, then decreased at TU stage and were very low at RR stage (Fig. 2d). Despite very low, the *COMT* transcript level in *hp-1* was significantly higher than in wild type at both MG and TU stage and may account for the higher accumulation of ferulic acid in *hp-1* peel at MG stage (Fig. 2d). Finally, sinapic acid had a similar content as in wild-type peel (Fig. 2c,d) with a very low level at RR stage (Fig. 2b,d). Similarly to wild-type and *hp-1*, caffeic acid was the most representative HCA in peel of other commercial cultivars, while the sinapic acid content was nearly negligible (Giuntini et al., 2008). Our previous analyses showed that a higher flavonoid and carotenoid content was correlated to a higher biosynthetic gene expression in *hp-1* compared to wild-type (Calvenzani et al., 2010; Lazzeri et al., 2012). In the present work, it was evident both in wild type and *hp-1* a higher accumulation of HCAs during the first stages of ripening (*i.e.* MG and TU stage; Fig. 2c,d), in contrast to what observed for flavonoids and

carotenoids, whose accumulation was much higher at TU and RR stage compared to MG stage (Calvenzani et al., 2010; Lazzeri et al., 2012). We may hypothesize that p-coumaric acid, a metabolic precursor common to both HCAs and flavonoid biosynthesis, may be initially channelled to HCAs synthesis at MG stage and then massively consumed by the flavonoid pathway in the subsequent TU and RR stages. Since HCAs are known to have a protective sunscreen role against UV-B rays in Arabidopsis (Jin et al., 2000), we may suppose that the accumulation of HCAs in the first stages of ripening could protect tomato fruits from UV-B during the first stages of maturation, when lycopene, β-carotene and flavonoids have not been accumulated yet (Calvenzani et al., 2010; Calvenzani et al., 2012; Giuntini et al., 2005).

Effect of UV-B depletion in peel of wild type and *hp-1*

In wild-type peel of UV-B-depleted fruits, no differences in the expression level were observed for *C4H, 4CL* and *C3H* between control conditions and UV-B depletion (Fig. 2a), whereas *PAL* showed a significant peak of transcript accumulation anticipated at MG stage (Fig. 2a) and *COMT* showed a very low peak of activation at TU stage (Fig. 2a). Although gene expression in wild-type fruits in UV-B-depleted conditions was similar to control conditions, HCAs content was generally lower and detected only at TU stage (Fig. 2c). This could not be explained by the fact that HCAs are the precursors of flavonoids, since flavonoid content was also reduced in wild-type peel in UV-B-depleted conditions as we have previously shown (Calvenzani et al., 2010). Since HCAs are also the precursors of lignins (Whetten and Sederoff, 1995), this reduction may be explained by an enhanced flux through this pathway. Only caffeic acid accumulated significantly more in UV-B-depleted than control conditions at TU stage; this increase was consistent with the significant decrease of its precursor p-coumaric acid at TU stage, although no differences in gene expression have been revealed (Fig. 2a,c).

In *hp-1* peel, compared to control conditions UV-B depletion determined a significant anticipated expression peak of all genes at MG stage instead of TU stage, except for *4CL* whose expression was

257 significantly diminished at MG stage (Fig. 2b). This enhanced gene expression somehow resulted in
258 a higher content of p-coumaric, caffeic and sinapic acids, all showing a significant peak of
259 accumulation at TU stage compared to MG stage. Low amount of ferulic acid may be due to a high
260 level of *F5H* transcript at MG stage, resulting in a 3-fold increase of sinapic acid with respect to
261 control conditions (Fig. 2b,d). Similarly to control conditions, both in wild-type and *hp-1* peel,
262 caffeic acid was the most abundant HCA in UV-B depleted conditions, even though in *hp-1* it
263 reached a 4-fold higher level than in wild type (Fig. 2c,d).
264 These data indicated that UV-B depletion have a different effect on the two genotypes. In wild-type
265 peel, despite no significant changes in the expression of HCAs biosynthetic genes, UV-B depletion
266 determined a general reduction of HCAs in accordance with Luthria studies (2006) in which the
267 total concentration of caffeic, coumaric and ferulic acids of UV-B depleted fruits was
268 approximatively 20% lower. On the other hand, in *hp-1* peel UV-B depletion generally exerted an
269 enhancing effect on both HCAs gene expression and accumulation. A different response to UV-B
270 depletion between the two genotypes was actually not unexpected. Previous studies indicated that
271 the influence of low UV-B radiation levels on ascorbic acid and lycopene levels (Giuntini et al.,
272 2005) and content of total soluble solids in fruits (Krizek et al., 2006) depends on tomato genotype
273 and that the results obtained may be valid only for the tomato cultivar studied (Papaioannou et al.,
274 2012). A genotype-dependence was observed also in two tomato hybrids characterised by a high
275 (DRW 5981) or a low lycopene (Esperanza) content, with DRW 5981 undergoing a decrease and
276 Esperanza an increase in HCAs content in peel following UV-B shielding (Giuntini et al., 2008).
277 However, when fruits of these two genotypes were subjected to post-harvest UV-B irradiation until
278 full ripeness, both exhibited an increased HCA accumulation in the peel, even if almost exclusively
279 in fruits collected at MG stage(Castagna et al., 2013), suggesting that UV-B responsiveness is a
280 highly regulated process, depending not only on genetic differences, but also on physiological stage.
281 Similarly to HCAs, our previous studies have indicated that UV-B depletion determined a severe
282 reduction of flavonoid content in wild-type peel, but differently from HCAs this reduction was

accompanied by a reduced flavonoid gene expression mostly at MG stage (Calvenzani et al., 2010). On the other hand, differently from HCAs, UV-B depletion did not greatly alter neither the content nor the flavonoid gene expression in *hp-1* peel (Calvenzani et al., 2010). These observations indicate that different branches of the phenylpropanoid pathway are differently regulated in response to UV-B, thus suggesting that the HCAs and flavonoid pathways in tomato may be controlled by branch-specific sets of regulatory genes. In Arabidopsis, the HCA biosynthetic pathway is induced by UV-B by repressing the expression of the *AtMYB4* regulatory gene (Jin et al., 2000), whereas the flavonoid pathway is activated by two different sets of regulatory genes, which control the early (*i.e. AtMYB11/12/111*) or the late (*i.e.* the MYB-bHLH-WD40 complex; (Petroni and Tonelli, 2011)) biosynthetic genes. In tomato, *LeMYB12* controls the flavonol synthesis by predominantly activating the early biosynthetic genes (Adato et al., 2009; Ballester et al., 2010), whereas *LeAN2* and *LeANT1* control the anthocyanin synthesis by activating the late biosynthetic genes (Povero et al., 2011; Sapir et al., 2008). However, no regulator of the HCAs branch in tomato has been identified so far.

HCAs content and gene expression in flesh of wild type and *hp-1* in control conditions

In wild-type flesh of fruits grown in control conditions, *PAL* was significantly more expressed at MG and TU stage compared to RR stage and *C4H* at TU stage compared to both MG and RR stage (Fig. 3a). The expression level of *4CL* significantly increased during ripening, whereas *C3H*, *COMT* and *F5H* significantly decreased after a peak at MG stage (Fig. 3a). In particular, the *COMT* transcript was no longer detectable at TU stage, whereas *C3H* and *F5H* were present at this stage even if at very low levels (Fig. 3a). The content of all HCAs analysed significantly increased during ripening in flesh of wild type fruits (Fig. 3c). In particular, the accumulation of caffeic acid, the most abundant HCA, may be due to the low level of *COMT* transcript, that decreased during ripening with a consequent lower consumption of its substrate (Fig. 1 and 3a,c). Accordingly, the amount of ferulic and sinapic acids was very low, despite they increased during ripening (Fig. 3a,c).

In *hp-1* flesh, *PAL* was not significantly different compared to wild-type, whereas *C4H* and *4CL* showed a significant anticipated peak at MG stage instead of TU or RR stage as in wild type. The *C3H, COMT* and *F5H* genes were more expressed at MG stage as in wild type, but the *COMT* and *F5H* transcripts reached a significantly higher level than in wild type (Fig.3a,b). Content of all HCAs analysed, except caffeic acid, showed an anticipated peak at TU stage which was significantly higher than at RR stage, unlike wild type, in which they reached higher level at RR stage (Fig 3c,d). This accumulation pattern may be due to the anticipated accumulation of *C4H* transcript at MG stage, with respect to wild type. Moreover, high levels of the *COMT* and *F5H* transcripts at MG stage may determine anticipated accumulation of ferulic and sinapic acid at TU stage.

In general, the expression level of the analysed genes was similar in wild-type and *hp-1* flesh in control conditions (Fig. 3a,b), whereas accumulation of HCAs in *hp-1* flesh was reduced with respect to wild type (Fig. 3c,d). This reduced accumulation of HCAs cannot be accounted by a significant difference of accumulation of the *PAL* transcript, coding for the enzyme that ensures the metabolic flux through the phenylpropanoid pathway (Fig. 3b) (Gonzali et al., 2009; Luo et al., 2008). However, our previous studies indicated that flavonoid content in *hp-1* flesh was up to 8.5-fold higher than in wild-type (Calvenzani et al., 2010). Since hydroxycinnamic acids are central compounds in the phenylpropanoid biosynthetic pathway (El-Seedi et al., 2012), we can hypothesize that the decreased content in HCAs with respect to wild type, in spite of a similar gene transcript level, may be due to a possible redirection of metabolic flux towards the flavonoid pathway, preventing the accumulation of HCAs.

Effect of UV-B depletion in flesh of wild-type and *hp-1*

In UV-B depleted conditions, in wild-type fruits, the *PAL* transcript resulted unchanged with respect to control conditions, whereas the *C4H* transcript level significantly increased at TU stage

and the *4CL* and *F5H* transcript level significantly decreased or increased at RR stage respectively (Fig. 3a). Interestingly, the *COMT* transcript was expressed at high level at TU stage (Fig. 3a). The content of all HCAs analysed in wild-type fruits was significantly reduced with respect to control, except caffeic acid whose content was comparable to control conditions at MG and TU stages, but diminished at RR stage (Fig. 3c). In particular, the decrease of ferulic and sinapic acids was consistent with a decreased expression of *COMT* and *F5H* at MG stage (Fig. 3a,c). The high level of *COMT* expression at TU stage may explain the higher amount of ferulic acid at RR stage, similarly to p-coumaric acid, whose increase may be due to the increase of *C4H* expression at TU stage.

Despite no data are presently available on the penetration of UV-B radiation in tomato fruits, these data and those from our previous studies (Calvenzani et al., 2010) indicated that both HCA and flavonoid biosynthesis genes are affected by UV-B depletion in flesh, suggesting that UV-B has a direct or indirect role in modulating gene expression in flesh.

In *hp-1* UV-B depleted fruits, *PAL* and *C4H* showed no significant difference compared to control conditions, whereas the *F5H* transcript level was significantly higher at MG stage (Fig. 3b). Also *4CL* and *COMT* showed an expression pattern similar to control conditions, but their transcripts resulted to be significantly decreased at MG stage (Fig. 3b). The *C3H* transcript level was unchanged at MG and TU stages in UV-B depletion, but significantly increased at RR stage with respect to control conditions, as observed in wild type (Fig. 3b).

In UV-B-depleted conditions, HCAs in *hp-1* flesh followed different trends of accumulation. Caffeic and p-coumaric acids increased during ripening and reached a significantly higher level with respect to control at RR stage. Caffeic acid accumulation was the highest of flesh in all analysed stages, whereas ferulic and sinapic acids presented a significant reduction at TU stage with respect to control conditions (Fig. 3d). The higher accumulation of caffeic acid presumably derived from the constant *4CL* expression along the ripening stages. Increased content of p-coumaric acid in comparison to control may be explained by the slight increase of *PAL* and *C4H* and the concomitant

decrease in *4CL* transcript accumulation. Similarly, reduced content of ferulic and sinapic acids could be explained by reduced transcript accumulation of *COMT* transcripts.

CONCLUSIONS

Our studies indicated a higher accumulation of HCAs in peel during the first stages of ripening in both genotypes, but comparing wild type and *hp-1* mutant, total HCAs content of peel was higher in the latter, as previously observed for flavonoids (Calvenzani et al., 2010) and carotenoids (Lazzeri et al., 2012), Moreover, UV-B-depletion response was genotype dependent as in our previous studies (Giuntini et al., 2008), and led to a lower HCA content in wild-type peel and flesh, as in commercial cultivars analysed by Luthria et al (2006), and higher in *hp-1* (Fig. 2-3c,d) generally in accordance with biosynthetic gene expression. Considering the similar trends shown by HCAs and flavonoids (Calvenzani et al., 2010), we can speculate that tomato SIDDB1 may be involved in the regulation of the HCA pathway by UV-B light during "*in planta*" ripening, although its expression was found not to be affected by UV-B light (Calvenzani et al., 2010).

The overall data indicate that different branches of the phenylpropanoid pathway are differently regulated in response to UV-B, thus suggesting that the HCAs and flavonoid pathways in tomato may be controlled by branch-specific sets of regulatory genes. Further studies will be necessary to understand the molecular mechanism underlying this different response.

ABBREVIATIONS USED

HCA, Hydroxycinnamic acid; UV-B, Ultraviolet-B; *hp-1*, *high pigment-1*; MG, mature green; TU, turning; RR, red ripe

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FIGURE CAPTIONS

Fig. 1 Hydroxycinnamic acids biosynthetic pathway. *PAL*, Phe ammonia-lyase; *C4H*, cinnamate 4-hydroxylase; *C3H*, *p*-coumaroyl ester 3-hydroxylase; *COMT*, caffeic acid 3-*O*-methyltransferase; *F5H*, ferulate 5-hydroxylase; *4CL*, 4-coumarate:CoA ligase (modified from Wink (2010)).

Fig. 2 Expression of HCA biosynthetic genes and HCA content in peel of wild-type and *hp-1* fruits grown in presence or absence of UV-B. Relative expression levels of the genes *PAL*, *C4H*, *C3H*, *COMT*, *F5H* and *4CL*, in the peel of wild-type and hp-1 fruits (a and b, respectively) using *LeEF1*

as endogenous control. The transcript amount in the flesh of MG fruits was arbitrarily set to 1 and it served as calibrator for relative expression levels in each transcript analysis.

Content of the different HCAs analysed in the peel of wild-type and hp-1 fruits (c and d, respectively) are expressed in mg/100 g of fresh weight.

White and black columns represent control fruits and UV-B-shielded fruits, respectively.

Bars represent standard errors of measurements performed in triplicate.

Abbreviations: MG, mature green; TU, turning; RR, red ripe

Fig. 3 Expression of HCA biosynthetic genes and HCA content in flesh of wild-type and *hp-1* fruits grown in presence or absence of UV-B . Relative expression levels of the genes *PAL*, *C4H*, *C3H*, *COMT*, *F5H* and *4CL*, in the flesh of wild-type and hp-1 fruits (a and b,respectively) using *LeEF1* as endogenous control. The transcript amount in the flesh of MG fruits was arbitrarily set to 1 and it served as calibrator for relative expression levels in each transcript analysis.

Content of the different HCAs analysed in the peel of wild-type and hp-1 fruits (c and d, respectively) are expressed in mg/100 g of fresh weight.

White and black columns represent control fruits and UV-B-shielded fruits, respectively.

Bars represent standard errors of measurements performed in triplicate.

Abbreviations: MG, mature green; TU, turning; RR, red ripe

Table 1. Primer Sets Used for Real Time RT-PCR

gene	primer name	Sequences (5'→3')
PAL	LePAL_TOM_F	5'-AACCTATCTCGTGGCTCTTT-3'
	LePAL_TOM_R	5'-TCTTTTTCGCTGAATCTTGC-3'
C4H	C4H -F1	5'-CCACAGAAAGGAGAGATCAACGAG3'
	C4H-R1	5'-CACAGCCTGAAGGTATGGAAGC-3'
4CL	4CL_TOM-F	5'-ACACACAAAGGCTTAGTCACGA-3'
	4CL_TOM-R	5'-AACAGAGGCAACACCACCACCATCA-3'
C3H	C3H-F1	5'-GGGTCACTTGTTGCATCATT-3'
	C3H-R1	5'-TGCAGGCAATCTTGGAGTAG-3'
COMT	COMT-F2	5'-CGACTTGGTCAAGATTGGTG-3'
	COMT-R2	5'-CCAACGCCTTGTTAAGTTCC-3'
F5H	F5H-F1	5'-GGATTGGAGAGGGACAAAAG-3'
	F5H-R1	5'-CAAACCATAGGAGGGGCAT-3'
EF1	EF1-F4	5'- GTTGGTCGTGTTGAAATGG -3'
	EF1-R3	5'-AACATTGTCACCAGGGAGTG-3'

Figure 1

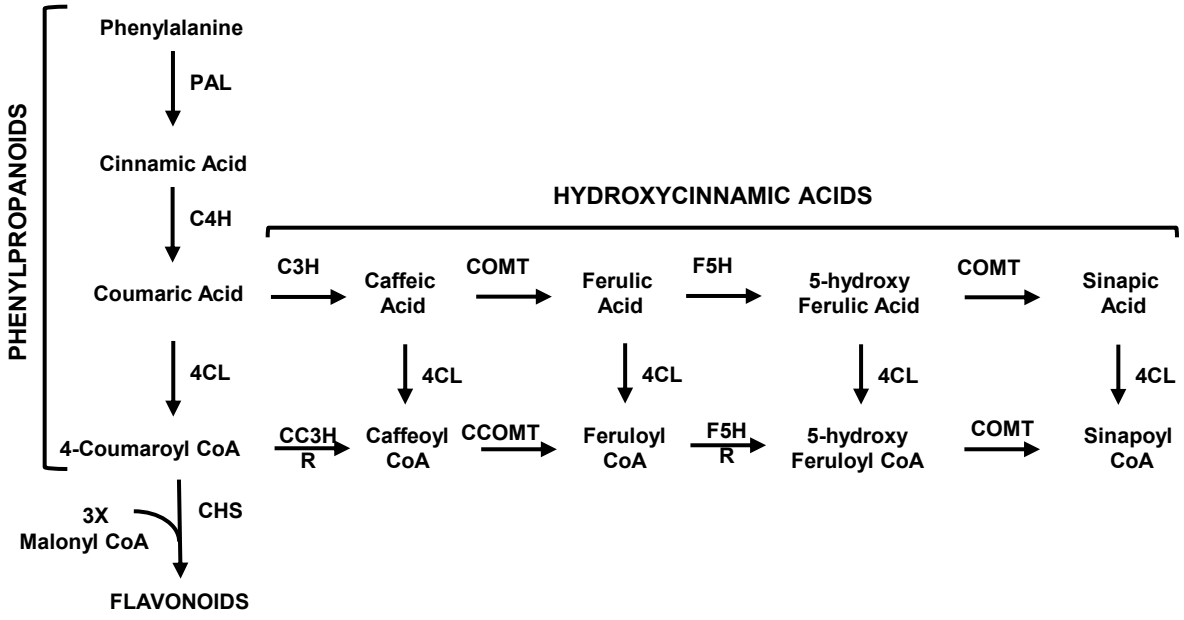

PHENYLPROPANOIDS
Phenylalanine
PAL
Cinnamic Acid
C4H
Coumaric Acid
4CL
4-Coumaroyl CoA
3X
Malonyl CoA
CHS
FLAVONOIDS
HYDROXYCINNAMIC ACIDS
C3H
Caffeic Acid
COMT
Ferulic Acid
F5H
5-hydroxy Ferulic Acid
COMT
Sinapic Acid
4CL
4CL
4CL
4CL
CC3H R
Caffeoyl CoA
CCOMT
Feruloyl CoA
F5H R
5-hydroxy Feruloyl CoA
COMT
Sinapoyl CoA

Figure 2

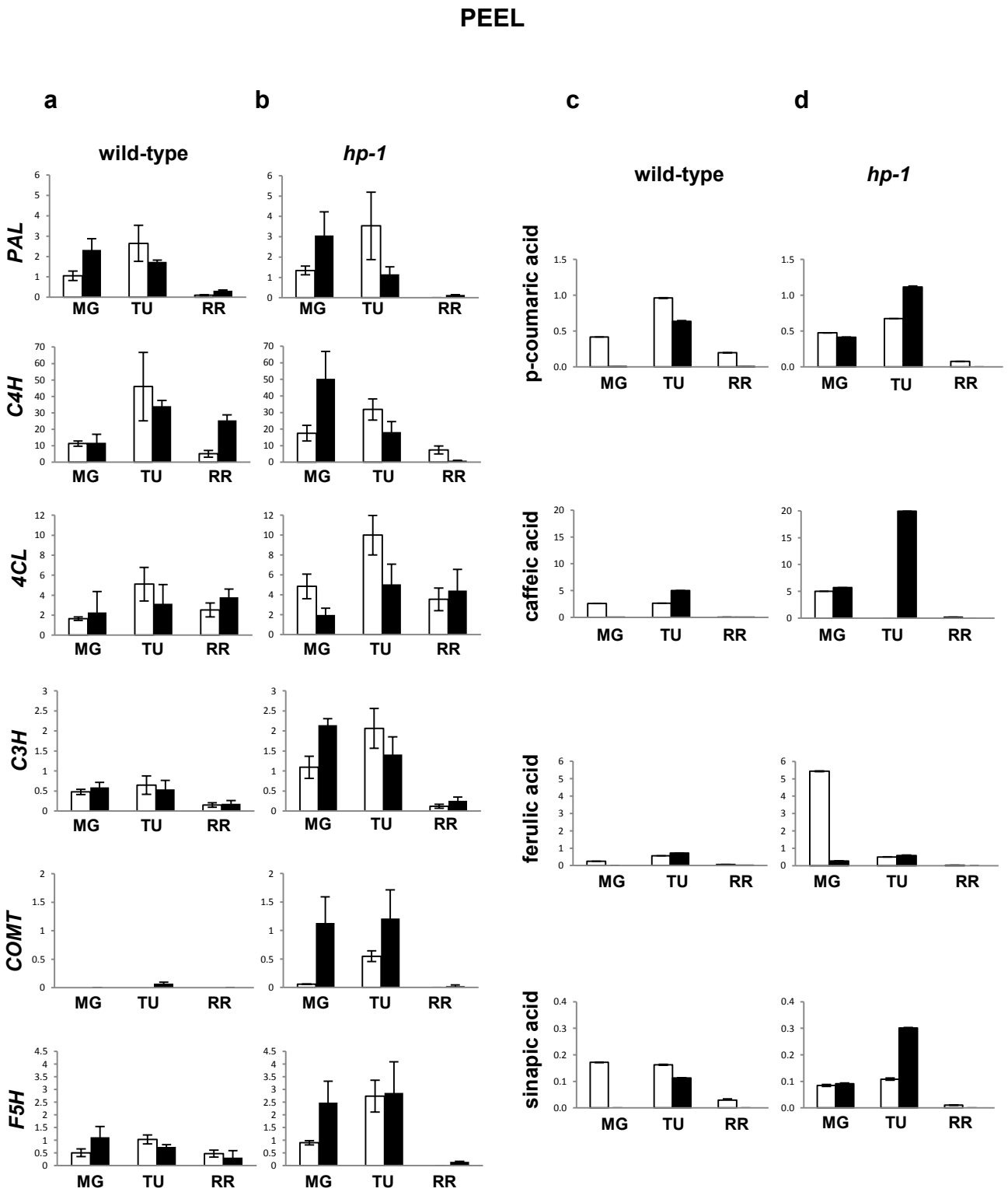
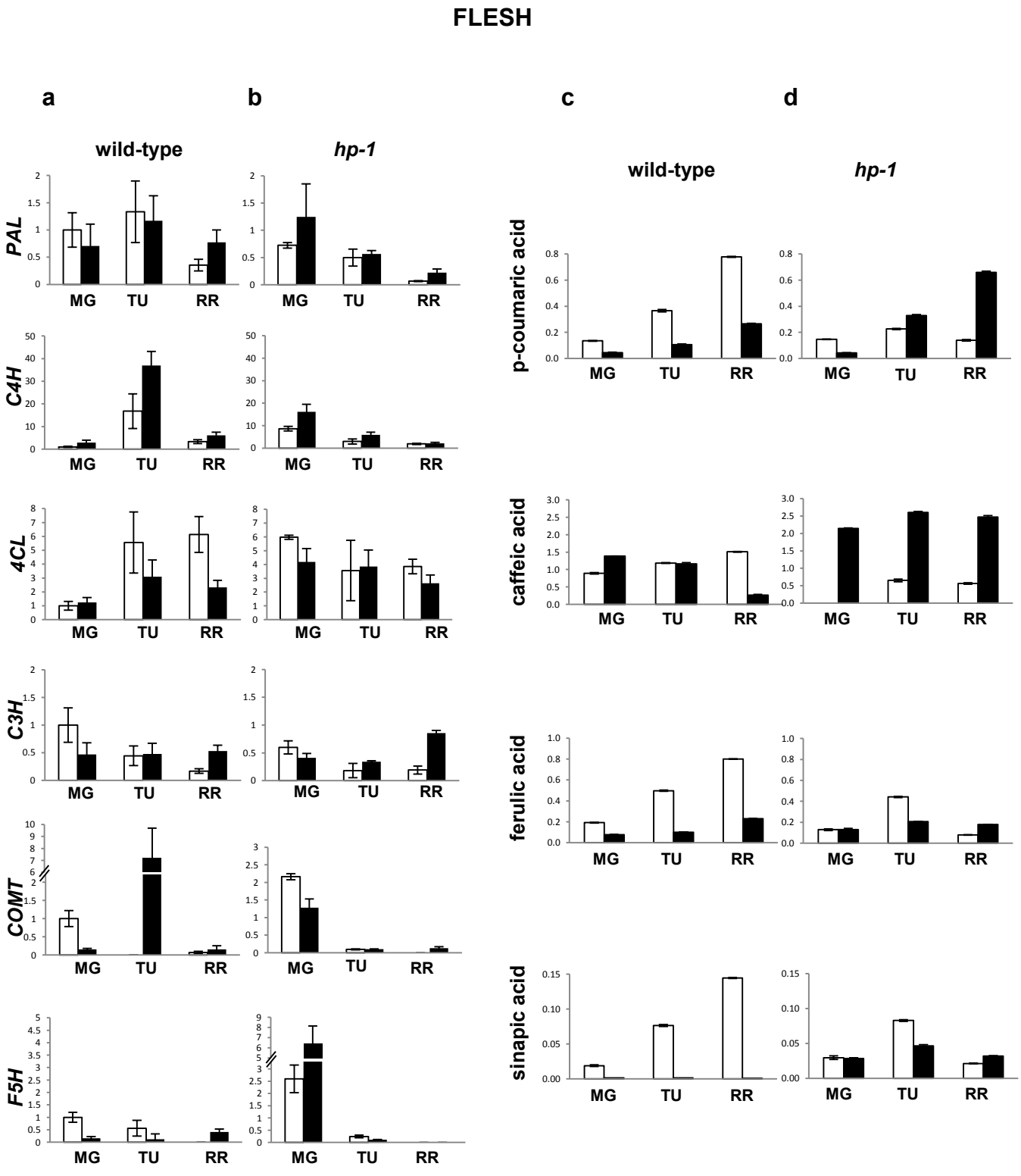

PEEL
a
b
c
d
wild-type
hp-1
wild-type
hp-1
PAL
C4H
4CL
C3H
COMT
F5H
p-coumaric acid
caffeic acid
ferulic acid
sinapic acid
MG
TU
RR

Figure 3


FLESH
a
b
c
d
wild-type
hp-1
wild-type
hp-1
PAL
C4H
4CL
C3H
COMT
F5H
p-coumaric acid
caffeic acid
ferulic acid
sinapic acid
MG
TU
RR