# Scrapped but not neglected: insights into the composition, molecular modulation and antioxidant capacity of phenols in peel of eggplant (*Solanum melongena L.*) fruits at different developmental stages

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**HIGHLIGHTS**

- Eggplant shows a dramatic change of phenolic composition during berry development
- Anthocyanins are substituted by chalcones at physiological ripening
- Transcript level of genes of the anthocyanin and chalcones pathways is in accordance with biochemical changes
- Antioxidant capacity of eggplant peel extracts slightly decreases along development

**ABSTRACT**

Eggplant fruits are normally harvested and marketed when they reach the commercial maturity, that precedes the physiological ripening when dramatic changes in taste, composition and peel color take place. The biochemical changes in fruit peel across the developmental stages, characterized also by a sizeable decrement of anthocyanins, were studied in four eggplant genotypes differing for fruit pigmentation. HPLC-DAD, HPLC-ESI-MS and NMR analyses identified naringenin chalcone and naringenin 7-*O*-glucoside as the main phenolic compounds in extracts from the physiological ripe stage, along with compounds tentatively identified as glycosylated naringenin chalcone, naringenin and kaempferol. On average, the levels of anthocyanins, responsible for the peel pigmentation, dropped by 75% during development, while, surprisingly, the level of total phenols showed a slight decrease of 16%, with a final concentration of more than 1000 mg/100g dw. RT-qPCR expression profiling of nine genes coding for enzymes putatively acting at different steps of the involved pathways showed modulation mostly consistent with the observed changes in phenolic composition, with a remarkable decrease in the activity of flavonol reductase and an increase in flavonol synthase during berry development. Antioxidant activity monitored by peroxyl scavenging was similar at all developmental stages while Fremy's analysis evidenced a slight decrement at full physiological ripening. These results are valuable to address the improvement of eggplant commercial fruit quality and the valorization of unmarketable physiological ripe fruits, especially for the newly accumulation of the health-promoting compounds chalcones and flavanones.

**Keywords**: fleshly fruit, *Solanaceae*, pathway of phenylpropanoids, anthocyanins, flavonoids, chalcones

## 1. INTRODUCTION

Phenols are widespread plant compounds considered important secondary metabolites with antioxidative activity and healthy properties (Shadidi and Naczk, 2019). The knowledge about these compounds in *Solanum* species has been mainly focused on their accumulation in edible organs (Simonne *et al*., 2011), and a special attention has been devoted to the berries of common eggplant (*Solanum melongena* L.), for their richness in phenolic compounds that have been ascribed to entail high antioxidant capacity (Akanitapichat *et al*., 2010). The fruits of *S. melongena* and allied species revealed a complex phenol composition, with different compounds accumulating in peel and flesh. The most significant classes of phenolic compounds identified in both tissues are hydroxycinnamic acids, mainly represented by caffeic acid (Wu *et al*., 2013), conjugated with organic acids such as quinic and malonic (Ma *et al*., 2010) and nitrogen-containing compounds, such as amides (Whitaker and Stommel, 2003). Another class of compounds highly accumulated in eggplant peel are anthocyanins, mainly present as glycosidic conjugates of delphinidin (Ichiyanagi *et al*., 2005), responsible of a variety of tonalities of black, lilac and purple colors which characterize various eggplant fruit typologies after its domestication (Azuma *et al*., 2008).

Many factors influence the qualitative and quantitative content of phenolic compounds in plants, among which the developmental stage is one of the most important (Heimler *et al*., 2017) and this is of great significance for fruit-bearing trees and vegetable crops. Extensive research on the changes in phenolic composition during ripening stages has been conducted in grape, both by chemical (Delgado *et al*., 2004) and by transcriptional approach (Gouthu *et al*., 2014). Similar studies have been extended to other fruits such as pepper and tomato, where changes across the various stages of the berry were reported and tomato is now becoming a model for the research on fleshy fruits (Calumpang *et al*., 2020).

In eggplant, the characterization of the process of berry development and subsequent ripening is lagging behind compared to tomato and pepper, whose genetic and molecular regulation were reviewed by Paran and van der Knaap (2007). In particular, the availability of mutants improved the knowledge of the tomatoes ripening process (Torres and Andrews, 2006). The eggplant fruit development is generally divided into three main stages: unripe, commercially mature and physiologically ripe (Mennella *et al*., 2012). In purple, lilac and black eggplant fruits, the stage of commercial maturation is empirically and visually evaluated based on the peel color tonality, intensity and brightness. Anthocyanin pigments accumulate to the highest levels at unripe fruit stage and decline as ripening progresses, with fruits turning from a glossy purple/lilac to brown/dark-yellow dull colors when they reach physiological ripeness (see **Figure 1**). Fruits at this stage lose their commercial value as they are characterized by a loss of color, and above all by presence of mature seeds and spoilage of the flesh texture. Eggplant fruits are therefore normally harvested well before the ripe stage when the colored cultivars have high anthocyanin levels still

reflected in strong purple, lilac or black pigmentations and seeds are barely detectable in the pulp, if at all (Rotino *et al*., 1997).

The studies about eggplant fruit development were focused on the accumulation of anthocyanin, sugars, phenolics and glycoalkaloids at commercial maturation, while there is a lack of knowledge on the molecular and metabolic changes leading up to physiological ripening. Mennella *et al*. (2012) reported a reduction of the two main classes of phenolic compounds (chlorogenic acid and delphinidin glycosides) in fruit peel during the transition from the commercially mature to physiologically ripe berries. Moreover, a recent study, aimed at evaluating the health properties of eggplant peel extracts, highlighted significant differences in the peel activity at the two different developmental stages (commercial and physiological), showing a significant anti-herpes activity only in extracts from physiologically ripe peels, associated to a noticeable increase of some flavonoids (Di Sotto *et al*., 2018). A better understanding of the molecular and biochemical aspects underlying the switch from the commercial mature to physiological ripe eggplant berries would therefore be of pivotal importance to extend the marketability of production and/or enable an exploitation of the ripe fruits.

In the present work, with the purpose to enhance the knowledge on the development of the eggplant fruit, we aimed at building a better understanding of the deep modifications in the peel biochemical composition over the various developmental stages, with a special focus on the later stages of ripening. Peel extracts of eggplant berries displaying different pigmentation were examined for their phenol content by HPLC-DAD and further evaluated for their antioxidant capacity by means of two *in vitro* assays. The phenol composition at late ripening stages was studied by fractionation of ethanolic extracts on $C_{18}$ and LH-20 resins followed by LC-ESI-MS, NMR and ESI-MS analysis, to identify the newly formed phenolic compounds. Moreover, expression levels of putative candidate genes involved in the biosynthesis and regulation of different phenolic compounds have been investigated.

## 2. MATERIALS AND METHODS

### *2.1 Plant material*

The genetic materials employed were the two parental lines '305E40' and '67/3' plus their hybrid (HF1) from which a F2 and Recombinant Inbred Lines populations have been obtained and used for QTL mapping, respectively, of biochemical (Toppino *et al*., 2016) and metabolic traits (Sulli et al, 2021). The female parent, '305E40', is an introgression line from *S. aethiopicum* bearing elongated dark purple fruits containing the anthocyanin delphinidin-3-rutinoside (D3R). The male parent, the line '67/3', whose genome has been recently sequenced (Barchi *et al*., 2019), bears round lilac fruits containing the anthocyanin nasunin (NAS) which is present also in the HF1. Furthermore, a fourth genotype analyzed was the 'L131'

line (Bianca Fus) which produces white round fruits and is almost deprived of anthocyanins in all its tissues and organs.

Thirty plants of each genotype were grown at CREA Montanaso L. (45°20'N, 9°26'E) experimental open field, distributed in three replications of 10 plants. The fruit ripening stages (**Figure 1**) were established as in Mennella *et al*. (2012) with a modification, and precisely: unripe stage A, approximately 21 Days After Flowering (DAF); commercially mature stage B, approximately 38 DAF; and ripe stage C, which in the present work was further divided in early (C1, approx. 60 DAF) and late (C2, approx. 80 DAF) ripeness stages. Fruits at the stage A have a tender and flexible peduncle, are still actively growing and are close to half their final size; their soft flesh is still greenish ('305E40' and 'HF1') or white ('67/3' and 'L131') and they are characterized by a glossy peel color, while seeds have not reached final size and display a white tegument. Fruits at the stage B have almost reached their commercial final size, displaying their typical peel coloration; in '305E40' and the HF1 hybrid the flesh is less greenish but shows a characteristic green ring next to the skin (absent in '67/3' and 'L131'), seeds have almost reached their final size but are still immature. The fruits harvested at the stage C had an increased firmness, the calyx and peduncle are quite lignified, the peel color appears brownish (C1) and dark brownish (C2) in '67/3', '305E40' and HF1, while it looks yellowish (C1) and dark yellowish (C2) in 'L131'. At the C1-C2 stages the flesh becomes progressively spongier with a white-yellowish color and it contains fully mature seeds.

The biochemical analyses were performed in triplicate using a pool of 10-15 eggplant berries from each field replication and fruit developmental stage, the harvested fruits were cleaned under tap water and immediately prepared by carefully trimming off the peel from each fruit with a lancet. Peel pooled samples were subsequently quickly frozen in a forced-air tunnel at -50 °C, and further lyophilized. After the lyophilization, the samples were powdered in a waring-blender at 4 °C and stored at -20 °C in dark glass bottles until analyses.

Molecular analyses were carried out on the highly homozygous lines '67/3', '305E40' and 'L131' at the three developmental stages A, B and C2. Very thin peel strips from at least three different fruits for each field replication and developmental stage were collected using a scalpel blade, pooled, and immediately frozen in liquid nitrogen, then stored at -80 °C.

### *2.2 Extraction and fractionation*

The initial extract from each replicate and developmental stage was obtained from 1 g of lyophilized powdered peel, treated with 15 mL EtOH/HCl 0.1 N 1:1. This mixture was shaken at room temperature for two hours, then centrifuged at 25000 × g at 4 °C for 20 minutes. The supernatants, referred to as "raw extracts", were collected by filtration through purified glass wool and stored at -80 °C until use for successive analyses.

As for the preparative separation of extracts at the ripening stage C2, an identical aliquot of powdered peel from each of the four genotypes assayed ('305E40', '67/3', 'HF1' and 'L131') was pooled and extracted for phenolics as described above (5 g with 75 mL). The solution was evaporated under vacuum at 40 °C, until the EtOH was evaporated, then four consecutive extractions were made with EtOAc saturated with water. The organic layer was separated by centrifugation and filtration on anhydrous $Na_2SO_4$. The EtOAc was evaporated under vacuum at 40 °C, until dryness, and subsequently re-dissolved in MeOH at a volume of around 20 mL ("semi-purified extract"). This extract, obtained in duplicate, was used for further steps of identification and fractionation (see after).

The fractionation of semi-purified extracts was performed into two steps, the first with $C_{18}$ resin, the second with LH-20 resin.

The first fractionation was performed on a column (1.8 cm diameter, 5 cm height) filled with 8 g of $C_{18}$ ICN Adsorbentien Biomedicals (32-63 μm, 60 Å) resin, preconditioned with 3 × 20 mL of MeOH 10% in water. The sample (3.5 mL), before elution, was 10-fold diluted with water, and was eluted at a flow rate of 1 mL/min. After washing with 2 × 20 mL of water, the column was subsequently eluted with 3 × 20 mL solutions at increased MeOH concentration, 30, 50 and 100%, to obtain fractions A, B and C, respectively. All resulting fractions were concentrated to small volume and re-dissolved with MeOH 50% in water, to a final volume of 5 mL.

Further separation was made by an elution on columns (1.2 cm diameter, 5 cm height) filled with Sephadex LH-20. The elution was conducted by gravity at room temperature at a flow of 0.7 mL/min after conditioning with MeOH 10% in water. Each $C_{18}$ fraction A, B and C, was 5-fold diluted with water and charged on the LH-20 columns at a volume of 5 mL. Each LH-20 elution was performed with 3 × 5 mL of the following solvents: for fractions A and B, with MeOH 10, 20, 30, 50 and 100%; for fraction C, with MeOH 70 and 100% and with MeOH/EtOAc 1:1. Each LH-20 fraction was concentrated at small volume to eliminate the organic solvents and stored at -80 °C in MeOH 50%. These purified fractions were further analyzed by HPLC, to evaluate their composition and the effectiveness of the solid-phase separation.

*2.3 HPLC-DAD analysis*

The HPLC analysis on raw extracts, semi-purified extracts and purified fractions was performed with a JASCO system equipped with a diode array detector (MD-2010 JASCO). The pump (PU-980 JASCO) was coupled with a quaternary gradient unit (LG-1580-02 JASCO). The analytical data were evaluated using a software-management system of chromatographic data (ChromNAV, Jasco, version 1.14.01). The separation was performed by a reversed phase, using a $C_{18}$ Purospher Star 250 × 4 mm column. The flow rate was 0.6 mL/min, the injection volume 15 µL, and the oven temperature was 42 °C. The mobile phase consisted of water with 0.5% of formic acid (solvent A) and acetonitrile acidified with 0.5% of formic acid (solvent B). The gradient was as follows (A/B): 95/5 0-5 min, from 95/5 to 80/20 in 10 min, 80/20 for 5 min,

from 80/20 to 55/45 in 10 min, 55/45 for 10 min, from 55/45 to 95/5 in 10 min, 95/5 for 9 min. Total analysis time was 59 min.

The amount of total polyphenols (TP) was given by the sum of the identified compounds, namely chlorogenic acid (CGA), delphinidin anthocyanosides (D3R and NAS), flavonol-type compounds (F), the flavanones naringenin (NAR) and naringenin derivatives (N), naringenin chalcone (NAR-CHA) and other chalcones (CHA). Results were expressed on dry weight (dw).

Peak identification was performed both by the direct comparison with commercial standards (CGA) or purified ones for anthocyanosides D3R and NAS. Moreover, compounds mainly present in C1 and C2 stages were quantified by the calibration with analogous commercial standards: flavanones with naringenin, chalcones with naringenin chalcone and flavonols with rutin, after a further confirmation of their spectral and chromatographic properties on the purified fractions and the comparison with previous literature data.

*2.4 HPLC-MS analysis*

HPLC-MS was performed with a Thermo Finnigan Surveyor LC pump equipped with a Thermo Finnigan Surveyor photodiode array detector set at λ 280 and 355 nm and interfaced with the ESI Thermo Finnigan LCQ Advantage spectrometer, using a Gemini RP $C_{18}$ column (250 × 4.6 mm, Phenomenex, Torrance, CA, USA). Elution solvents and gradient conditions were adapted from those used with the Purospher column (see above).

*2.5 NMR and ESI-MS analysis*

$^{1}$H-NMR and $^{13}$C-NMR spectra were acquired at 400.13 MHz and 100.61 MHz, respectively, on a Bruker Advance 400 spectrometer (Bruker, Karlsruhe, Germany) interfaced with a workstation running Windows operating system and equipped with a TOPSPIN software package. Chemical shifts (ppm, δ) were referenced to solvent signal ($CD_3OD$ $\delta_H$ 3.35 and $\delta_C$ 49.3 ppm from TMS).

ESI-MS spectra were recorded on a Thermo Finnigan LCQ Advantage spectrometer (Hemel Hempstead, UK). Mass spectra were acquired in both positive and negative modes over a range *m/z* 100-1000.

*2.6 Molecular phylogenetic analysis by neighbor joining method*

Evolutionary analyses of peptide sequences were conducted in MEGA X (Kumar *et al.*, 2018). The evolutionary history was inferred using the Neighbor-Joining method with a bootstrap test (500 replicates). The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

*2.7 RNA extraction and RT-qPCR analysis*

Total RNA from all the eggplant peel samples was extracted using the TRIzol RNA Isolation Reagents (Thermo Fisher Scientific) combined with the Spectrum Plant Total RNA kit (Sigma Aldrich), each sample was resuspended in a final volume of 30 µL. From the total RNA extracted, 8 µL were treated with the RQ1 RNase-Free DNase Kit (PROMEGA). The single strand cDNA was synthesized from 1 µg of RNA using the ImProm-II™ Reverse Transcription System Kit (PROMEGA). The standard procedure was changed by adding 0.5 µg/reaction of both Oligo(dT) and Random Primers to 1 µg of DNase treated RNA and heating at 70 °C for 5 min. The RNA mix was cooled to 25 °C and 15 µL of RT Master Mix was added, prior to heating at 42 °C for 1 h and then 75 °C for 10 min, to inactivate the Reverse transcriptase. Primers for RT-analysis of the genes *Chalcone synthase* (*SmCHS_synthase0*, *SmCHS_ch05*), *Chalcone isomerase* (*SmCHI_ch10, SmCHI_ch05*), *Tetrahydroxychalcone-2'-glucosyltransferase* (*SmGT_ch01, SmGT_ch05, SmGT_ch10*), *Dihydroflavonol-4-reductase* (*SmDFR_ch00*) and *Flavonol synthase* (*SmFLS_ch04*) (detailed in **Supplementary Table 1**) were designed basing on the available eggplant genome sequence of '67/3' (Barchi *et al*., 2019; www.solgenomics.com) utilizing Primer 3 (https://primer3.org, coding sequence of the gene in **Supplementary Data 1**). The RT-qPCR reactions were carried out according to the following PCR parameters: 95 °C for 5 min, followed by incubation for 15 s at 92 °C and denaturation for 15 s at 95 °C, annealing for 60 s at 59 °C for 40 cycles, followed by elongation at 72 °C for 20 s. The reaction was performed using GoTaq® RT-qPCR Master Mix (PROMEGA). The reaction contained 1.0 µL of previously diluted cDNA (1:20), from 0.2 µL to 1.0 µL of primers (1 µM each), 5 µL of GoTaq® RT-qPCR Master Mix and RNase-Free water up to the final volume of 10 µL. All samples were run in three technical replicates, and no-template controls were included in all analyses. Standard curves for each primer pair were calculated across a 5-fold dilution series of pooled diluted cDNA amplified in technical triplicate. The PCR efficiency was calculated by Rotor-Gene 6000 Series Software and it was optimized to be in the range 90-100% with $R^2$-values of 0.996. The relative quantification of gene expression was performed using the geometric averaging method. Specificity of amplifications was assessed first by PCR for the presence of a single band and then through the melt curves analysis. The analyses were performed on three biological replicates and in technical triplicates. The relative expression ratio was calculated using the "Delta-delta method" using *SmelGAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) and *Smel18S* as housekeeping genes.

*2.8 Antioxidant capacity measurements*

The antioxidant capacity potential was measured by the Fremy's salt radical quenching and by the peroxyl radical ($ROO^{\bullet}$) scavenging in a system lypoxigenase-linoleic acid. The measurements of Fremy's salt scavenging were made by Electron Paramagnetic Resonance (EPR) using a MiniScope MS200 Magnettech (Berlin, Germany), while the $ROO^{\bullet}$ scavenging by a spectrophotometer set at 234 nm (JASCO V-630).

The Fremy's salt is a commercially available free radical compound that is soluble and stable in aqueous solutions. The scavenging reaction system contained 0.4 mL 100 mM phosphate buffer solution, pH 7.3, 0.1 mL extracting solution and 0.08 mL of 3.75 mM Fremy's salt dissolved in 100 mM phosphate buffer solution, pH 7.3. The test solution was prepared as above but substituting the 0.1 mL of extracting solution with the same volume of the eggplant raw extract. These solutions were accurately mixed in a glass tube assay and successively placed in a 50 µL capillary for EPR measurements. The experimental conditions of the spectrometer were: field set, 3350 G; scan range, 50 G; scan time, 120 s; modulation amplitude, 2000 mG; microwave attenuation, 7 dB; receiver gain, 7 × 10. EPR spectra were recorded after 1 min of reaction at 25 °C.

Antioxidant potential toward $ROO^{\bullet}$ was based on the inhibition of diene formation induced by the lypoxigenase action on a linoleate solution. A blank solution contained 2 mL of a 100 mM phosphate buffer solution, pH 7.4; 0.2 mL of 3.2 mM linoleate solution, pH 9.2; 50 µL of extracting solution and 25 µL of soybean lypoxigenase, 62400 U/mL. The solution with the tested scavenging compound was composed exactly as the blank, by replacing 50 µL of extracting solution with the same volume of eggplant extract. In these conditions, the delta absorbance of the blank solution at 234 nm between 30 and 90 s of reaction at 20 °C was around 1 AU.

For both tests, the calibration was made with CGA solution at known concentration, so the results were given as equivalents of CGA, the most representative phenol compound in eggplant.

### *2.9 Statistical Analysis*

For biochemical data, Statgraphics software ver. 5.1 (Manugistics, Rockville, Maryland, USA) was used to perform the multifactor ANOVA on phenol compounds concentrations, eggplant genotypes and ripening stages, and comparison of means was performed by means of Tukey HSD test ($P \leq 0.05$). In order to highlight the main contributors to the measured antioxidant capacity, the concentrations of phenol compounds were correlated by simple regression with their respective antioxidant parameters.

For gene expression data, statistical analysis was performed with JASP software (https://jasp-stats.org/) and the graph realized with GraphPad Prism 9. One-way ANOVA with Tukey's HSD post hoc test was performed.

## 3. RESULTS

### *3.1 Phenolic composition of peels*

The peel composition at stages A, B, C1 and C2 regarding the total phenols content (TP), chlorogenic acid (CGA) and anthocyanins (ATH) is reported in **Table 1**. No delphinidin derivative was present in the fruits of line 'L131', characterized by a white peel, and different forms of the delphinidin derivative characterized

the other assayed genotypes (delphinidin-3-rutinoside, [D3R] in '305E40'; nasunin, [NAS] in both '67/3' and 'HF1'). Taking into consideration the transition from the commercially mature (stage B) to the physiologically ripe fruit (stages C1 and C2), the content of CGA and ATH decreased dramatically in the pigmented genotypes. On average, CGA and ATH accounted for most of the TP in stages A and B, with their sum representing 93% and 88% of the TP in each stage, respectively. The average content of CGA varied from the maximum in stages A and B (664.6 and 624.7 mg/100g dw) to the statistically lower values of 397.3 and 246.5 mg/100g dw in C1 and C2 ripening stages, showing an average percent decrease of 61% between B and C2 (**Table 1**). Significant variation was also evidenced among all the tested genotypes, with '67/3' having an amount of CGA significantly higher than the others. Also, both delphinidin anthocyanosides D3R and NAS displayed a similar behavior, showing a significantly higher average content in the ripening stages A and B (745.7 and 548.8 mg/100g dw, respectively), dropping to an average of 139.8 mg/100g dw in stage C2, with an overall percent decrease of 75% between stage B and C2. Line '305E40' had a significant higher amount of the anthocyanin D3R with respect to the amount of NAS detected in '67/3' and HF1. This was especially evident in the unripe stages A and B, while in C2 the difference was reduced (e. g. ATH amount 150% higher in '305E40' compared to '67/3'at stage B, but only 17% higher at stage C2).

The average TP content showed a slight decrement along the developmental stages, which was statistically significant only when comparing the physiological ripe C1 stage to the stage A. By comparing the tested genotypes, a significantly lower TP content was evidenced in 'L131' with respect to lines '67/3' and '305E40'.

TP averaged, respectively, 1512.5 and 1119.9 mg/100g dw at stages A and C2 with a less dramatic decrease compared to that of either CGA or ATH, dropping only by 16% from stage B to stage C2 (**Table 1**). In fact, a number of additional phenolic compounds was detected specifically at the ripe stages, that compensated for the drop of CGA and ATH in the TP amount.

Since the detected phenols exhibit characteristic UV maxima, the first approach to identify these compounds was by HPLC-DAD chromatography of a semi-purified extract, where the main components resulted detectable at 280 and 355 nm (**Figure 2**). This analysis revealed a significant change in composition which took place in a similar fashion in all the tested genotypes when the fruit entered the stage of physiological ripeness C1 and C2. In particular, peel extracts sampled at the ripening stage C2 were characterized by the presence of distinctive compounds, present only in very low amounts in unripe and commercially ripe berries. At λ 280 nm (**Figure 2, top**), the compound with Rt about 37 min was identified with a high degree of confidence as it co-eluted with a commercial standard of naringenin (NAR), based on its UV-VIS spectra. Two other components, N1 and N2, presumably belonging to the flavanone family like NAR, are probably NAR derivatives conjugated with polar compounds that would explain their lower

retention times. The compound N2 was notably more abundant than N1 and NAR, thus it was purified for further identification (**Supplementary Figure 1**).

The chromatogram registered at λ 355 nm (**Figure 2, bottom**), revealed the presence of a main component eluting at around 36 min. It showed a characteristic UV-VIS spectrum, with the maximum absorbance peak at λ 368 nm. It perfectly overlaps with a peak of a tomato peel extract, having the same UV-VIS spectrum, identified as the naringenin chalcone (NAR-CHA) in previous studies (Muir *et al*., 2001); this compound was purified in order to confirm its structure (**Supplementary Figure 2**).

Three other compounds, indicated in **Figure 2** as CHA1, CHA2 and CHA3 (with CHA2 and CHA3 at very close Rt, and eluted before NAR-CHA), were tentatively attributed to glycosylated chalcones on the basis of their UV-VIS spectra (**Supplementary Figure 3**). Finally, a compound eluting at 29.0 min (**Figure 2, bottom**), with an UV-VIS spectrum peaking at 355 nm (**Supplementary Figure 3**), was tentatively identified as a flavonol-type derivative and named F1.

To get a better insight into the composition of the eggplant physiologically ripe peel, the semi-purified extract was submitted to further fractionation through SPE-$C_{18}$ resin followed by LH-20 separation. Four fractions were obtained containing the most relevant compounds in eggplant peel, namely:

- compound N2, found in fraction B eluted in MeOH 30% on LH-20 resin, having an UV-VIS spectrum very close to that of pure NAR (**Supplementary Figure 1**);
- NAR-CHA, found in fraction C eluted with MeOH/EtOAc 1:1, with an UV-VIS spectrum and a chromatographic behavior fully close to that of NAR-CHA **(Supplementary Figure 2**);
- compound CHA3, found in fraction B eluted in MeOH 100% on LH-20 resin, having an UV-VIS spectrum very close to that of pure NAR-CHA (**Supplementary Figure 3**);
- compound F1 found in fraction B eluted in MeOH 50% on LH-20 resin, having an UV-VIS spectrum related to a flavonol type compound (**Supplementary Figure 3**).

The eggplant peel extract was also analyzed with a HPLC ESI-MS apparatus coupled to a photodiode array detector set at λ 280 and 355 nm, with an elution system analogous to that of HPLC-DAD analysis. The chromatographic profiles obtained from HPLC-DAD-ESI-MS analysis with UV absorbance set at λ 280 nm and 355 nm **(Figure 3**), showed slight variations with respect to HPLC-DAD chromatograms (**Figure 2**). The chromatographic profiles, acquired by ESI-MS spectra in both positive and negative mode, compared with data from the literature, enabled to tentatively identify nine compounds (**Table 2**).

In particular, the compound eluting at Rt 26.03 min (N2), showed molecular ions m/z 434.70 and 433.21 in positive and negative modes, respectively, and fragmental peaks representing loss of one sugar from the molecular ion (at *m/z* 272.96 [M + H –Glc] and *m/z* 271.95 [M - H –Glc]). These data, together with the previously discussed UV absorbance maxima, allowed to tentatively identify the compound N2 as naringenin 7-*O*-glucoside (**Figure 4**) (Blunder *et al*., 2017; Wu *et al*., 2013). As for peak with Rt 21.7 min, the presence of a molecular ion at m/z 596,94 and fragments at *m/z* 434.79 [M-162] and 272.95 [M- 2 × 162]

due to the sequential loss of two hexose moieties, suggested for compound N1 the structure of a naringenin diglycoside.

Peaks corresponding to compounds CHA2 and CHA3, overlapping under the current chromatographic conditions, both showed a deprotonated molecular ion at *m/z* 433 and the aglycone ion at *m/z* 271 ([M-H-162], loss of a hexose moiety) and were tentatively characterized as glucosides of NAR-CHA, already identified in other plants (Iwashina and Kitajima, 2000; Cioffi *et al*., 2003). Their structure could be probably attributed to 2′ and 4′ glucosyl derivatives (**Figure 4**), from the similarity with elution profiles of Togami *et al.* (2011). Their aglycone (NAR-CHA), eluting at 32 min, presented the $[M-H]^-$ and $M + H]^-$ ion at *m/z* 271.22 and 272.95, respectively, and produced fragment ions at *m/z* 151.12 (and 152.87).

Peaks at 25.5 min (compound F1) and 27.0 min were identified as kaempferol derivatives on the basis of their characteristic fragmentation. In fact, both exhibited the same aglycone ion at *m/z* 286 due to the loss of 162 Da (a hexose residue) for the former and 248 Da (162 + 86 Da), corresponding to a malonyl hexose residue, for the latter. Thus, compound F1 is presumably a kaempferol glycoside and peak at 27.0 min its malonyl derivative, as malonylation of sugar moieties is a common feature in flavonoids (Katsube *et al*., 2006; Kamata *et al*., 2008). Finally, peak at 30.1 min showing the $[M + H]^+$ at *m/z* 289 and a fragment ion at m/z 152 was tentatively identified as dihydrokaempferol (aromadendrin). Such peaks at 27.0 and 30.1 minutes were not quantified during HPLC-DAD analysis (**Table 2**).

To achieve the definitive evidence that the most abundant components of the eggplant ripen peel extract are naringenin 7-*O*-glucoside (N2) and naringenin chalcone (NAR-CHA), peaks at 26.0 min and 32.3 min, corresponding to the most intense signals in the chromatograms recorded at λ 280 and 355 nm, respectively, were isolated by semipreparative SPE (**Supplementary Figures 1, 2** and **3**) and their structures further confirmed by NMR and ESI-MS analysis. In both cases, all spectral data are in full agreement with those reported in the literature (Blunder *et al*., 2017; Iwashina and Kitajima, 2000).

After the identification of the main eggplant peel phenylpropanoid compounds at the physiological ripening stage, the semi-quantification of the three flavanones N1, N2, NAR and of the flavonol-type compound F1 (overall indicated as FLAV), and of the four chalcones CHA1, CHA2, CHA3, NAR-CHA (overall, CHALC) was performed to characterize the changes in phenol composition correspondent to the different developmental stages in the four assayed lines (**Table 3).** The comparison of FLAV and CHALC compounds, characteristic of C1 and C2 ripening stages, revealed a clear opposite trend with respect to CGA and ATH, with increasing contents in physiologically ripe stages C1 and C2, especially in the latter one. FLAV content at the C1 ripening stage slightly increased but was not statistically different from previous stages, whereas it was significantly higher at the C2 ripening stage (**Table 3**). All the genotypes behaved in a similar way along fruit development, with a marked increase in C2 stage and no difference in the average FLAV content. The average percent increment of FLAV compounds was 120% in ripening stage C2 when compared to the stage B, with a range from 71% (‘L131’) to 160% (‘305E40’).

The CHALC compounds displayed a dramatic increment coincident with the shift from the commercial maturation to the physiologically ripe stages (**Table 3**). The average amount detected was similar at stage A and B and showed a significant increase in both stages C1 and C2. 'L131' accumulated a significant higher CHALC content at stage C2 than the other genotypes (296.2 mg/100g dw), among which '67/3' had the lower amount. The average increment of the CHALC compounds in the stage C2 over the stage B was of 642%, with a minimum of 171% ('67/3') and a maximum of 970% ('L131').

As for the main FLAV and CALC single compounds, (F1, N2 and NAR-CHA) a similar trend in the average concentrations was evidenced in pooled samples of the assayed genotypes (**Figure 5**) with a content statistically higher in the physiological ripening stages (C1 and/or C2) with respect to the earlier ones. On the contrary, NAR showed a minimum content in ripening stage C1 only significantly different from that of stage A.

*3.2 Identification and transcriptional profile of candidate genes for phenylpropanoid enzymes in peel during fruit ripening*

To monitor the expression of genes likely involved in determining the differences in phenolic composition in eggplant peel along the developmental stages, especially during the switch from stage B to C, the following candidate genes for key biosynthetic steps were chosen: *CHS* (Chalcone synthase) and *CHI* (Chalcone isomerase), responsible, respectively, for synthesis and isomerization of NAR-CHA, *GTs* (Glucosyltransferases), potentially using NAR-CHA and NAR as substrate for glucosilation, *DFR* (Dihydroflavonol-4-reductase), involved in the biosynthesis of ATH and, finally, *FLS* (Flavonol synthase) a key enzyme for the biosynthesis of flavonols (**Figure 4**).

*DFR* is widely reported as one of the key Late Biosynthesis Genes (LBGs) in the anthocyanin pathway in many Solanaceous species (Liu *et al*., 2018). The dihydroflavonol represent a branch point in flavonoid biosynthesis, being the intermediates for production of both the colored anthocyanins through the action of DFR, and the flavonols produced by FLS (Davies *et al*., 2003). To select the remaining genes, we explored RNA-seq information from previous work on '67/3' (Barchi *et al*., 2019) excluding those showing low levels of expression in fruit tissues and choosing two *CHS* (*SMEL_005g227980*, SMEL_000g090720) and *CHI* (*SMEL_010g353630*, *SMEL_005g226510*) for analysis. To select the GT genes of interest, we first carried out homology searches against the eggplant genome using sequences of proteins reported to catalyze the transfer of glucose from UDP-glucose to the 2'-hydroxyl group of chalcone to generate chalcone 2'-*O* glucoside (**Figure 4**), which confers a stable yellow color to the petals of carnation, cyclamen, and catharanthus, due to the lack of isomerization to colorless NAR (Ogata *et al*., 2004; Togami *et al*., 2011). All eggplant peptide sequences (**Supplementary Data 2**) obtained from the search with *e* value below $e^{-100}$ were included in a Neighbor Joining analysis to establish their relationship with the previously described sequences (**Figure 6**). SMEL_001g119020 was selected for being close to the four carnation GT sequences

and strongly expressed in '67/3' in fruit at ripening stage C (Barchi *et al*., 2019), while SMEL_010g340790 was included in the analysis for its homology to *Catharanthus* AB294401 and an intermediate level of expression in fruit. Finally, it was decided to test the expression of the GT SMEL_005g234820 as representative of a group of genes highly homologous to SMEL_001g119020 but predicted to be poorly expressed in the '67/3' fruit.

To investigate the expression of the identified genes *SmelCHS_ch00, SmelCHS_ch05, SmelCHI_ch10, SmelCHI_ch05, SmelGT_ch01, SmelGT_ch05, SmelGT_ch10, SmelDFR_ch00* and *SmelFLS_ch04*, we performed RT-qPCR expression analysis using peel samples from unripe (stage A), commercially mature (stage B) and fully physiologically ripe (stage C2) fruits of the lines '67/3', '305E40' and 'L131'. The pathway and biosynthetic steps where these genes are putatively involved are summarized in **Figure 4**.

The two *CHS SmelCHS_ch00* and *SmelCHS_ch05* are putatively orthologs of genes widely described in literature to encode enzymes synthesizing NAR-CHA starting from coumaroyl-CoA and malonyl-CoA. The expression profile of these two putative genes showed a common trend of expression in both the anthocyanin pigmented phenotypes '67/3' and '305E40' (**Figure 7**). A comparable expression level between the developmental stages A and B was detected for *SmelCHS_ch00*, followed by a significant increase in stage C. Interestingly, the anthocyanin-less genotype 'L131' also showed a dramatic increase in the expression of *SmCHS_ch00* at stage C (**Figure 7**). In this genotype the same trend was observed also for *SmCHS_ch05,* in contrast to the pigmented genotypes where the peak of expression for this gene was at stage A. The two *CHI* (*SmelCHI_ch10* and *SmelCHI_ch05)* putatively responsible for the isomerization of NAR-CHA, resulted clearly expressed in the pigmented fruits at stage A while not or weakly expressed at the stage B and C (**Figure 7**). By contrast, in 'L131' a significantly different level of expression was detected for *SmelCHI_ch05* only at stage C, while expression of *SmelCHI_ch10* was not detected at any developmental stage. The expression profile of the three putative *GT* genes in the pigmented genotypes confirmed high *SmelGT_ch01* expression levels at stage C, while *SmGT_ch05* was mainly expressed at stage A (**Figure 7**). *SmGT_ch10* resulted not detectable (data not shown). A similar trend to that observed in the pigmented lines was evidenced in 'L131' for *SmelGT_ch01* and *SmelGT_ch10*. By contrast, in this genotype *SmelGT_ch05* had the highest expression at stage C, showing a trend comparable to *SmelGT_ch01* similarly to *SmelCHS_ch05* (**Figure 7**). *SmelDFR_ch00* was only expressed in the pigmented genotypes at stage A, while no expression was detected in the 'L131' line. Finally, *SmelFLS_ch04* was strongly expressed at stage C in all the genotypes, showing an opposite trend with respect to *SmDFR_ch00*.

### *3.3 Antioxidant capacity*

Two *in vitro* antioxidant measurements were carried out on eggplant peel extracts of the four genotypes, the results are shown in **Figure 8**. A general decrease in antioxidant capacity was evidenced along the developmental stages. A flatter trend was given by the peroxyl scavenging, with values decreasing but non

significantly different from the maximum in stage A (**Figure 8a**). The Fremy's salt scavenging gave a clear decrease very similar to those of CGA and ATH, with the two developmental extremes A and C2 being significantly different (**Figure 8b**). The values of correlation, measured by simple regression, among the phenol compounds and the two antioxidant assays highlighted the close relationship between these parameters. The two assays were in a good correlation ($r^2$ = 0.434, $P < 0.05$), and an identical high correlation was found for the peroxyl scavenging with TP ($r^2$ = 0.482, $P < 0.01$) and for Fremy's salt scavenging with CGA ($r^2$ = 0.482, $P < 0.01$). On the other hand, for both antioxidant assays, no significant correlation was found for CHALC and FLAV, or for the most relevant single compounds NAR-CHA, NAR, N2 and F1.

## 4. DISCUSSION

In this work we measured the content changes of TP, CGA and ATH across four eggplant developmental stages (**Table 1**) and found trends comparable to those reported in previous works on lines of the same typologies in the same environment. In fact, Mennella *et al*. (2012) evidenced higher amounts of CGA at stage A (2319 mg/100g dw) in whole eggplant fruits, which decreased to 1482 mg/100g dw at stage B of ripening while the levels of ATH in peel averaged 1274 and 539 mg/100g dw at stage A for D3R and NAS, respectively; other works reported a wide range of ATH concentrations in eggplant peel (from 52 to 1975 mg/100g dw) as reviewed by Nino-Medina *et al*. (2017). Whitaker and Stommel (2003) found a wide variation of hydroxycinnamic acids content in different fruit samplings of various commercial eggplant varieties, with a range of 237-1474 mg/100g dw. The data presented here fall in these given ranges. Furthermore, our results are in accordance with those of a previous investigation by Wu *et al*. (2013) on stage A of eggplant berries from several accessions, where some chalcones, flavanones and flavonols were also found in the metabolic profile, using reversed-phase as chromatography and LC-TOF-MS as detection technique.

In a large number of species, the accumulation of anthocyanins lasts across all the late ripening stages as in pigmented grape and blueberry (Boss *et al*., 1996; Li *et al*., 2020). On the contrary, in *S. melongena* the ATH pathway is differently regulated and stops or dramatically drops down at the onset of physiological ripeness. Our results show how the dramatic decrease of ATH at physiological ripeness, accompanied by a drop in CGA levels, does not reflect in a dramatic TP decrement, as an increase in flavanones and flavonols also takes place at this stage. As a result, the TP content of commercially and physiologically ripe fruits is quite similar (**Tables 1** and **3**), as well as their peroxyl antioxidant capacity (**Figure 8a**). Indeed, *CHS* genes are expressed in eggplant fruits peel at all stages (**Figure 7**), suggesting that the production of NAR-CHA is maintained throughout maturation and shows a peak of expression at the stage of physiological ripening C2. By contrast, the observed drop in expression of the *CHI* genes at stages B and C2 may be indicative of a

reduced conversion of NAR-CHA into NAR as maturation progresses, suggesting a consequent switch towards alternative branches of the phenol biosynthesis pathway.

The highlighted biochemical changes point at a lowering content of ATH throughout fruit maturation, which is accompanied by a progressive increase of NAR-CHA and naringenin 7-*O*-glucoside (**Tables 2** and **3**), resulting the most abundant polyphenols in peel at ripening stage C2. To the best of our knowledge, we report for the first time both the identification and quantification of naringenin 7-*O*-glucoside (N2) and NAR-CHA as the more representative phenol compounds in the peel of physiologically ripe eggplant. In fact, no quantitative data of these compounds were found in literature, and qualitative detection was reported only for earlier stages of maturation (Wu *et al*., 2013; Calumpang *et al*., 2020).

The increment of FLAV and CHALC compounds is in agreement with the modulation of expression of most of the genes analyzed. The increase of *CHS* transcripts at stage C2 agrees with the CHS key function, as it codes for an enzyme at beginning of the pathway (**Figure 4**), as observed by Wang and Fu (2018) who reported that virus-mediated *CHS* silencing ultimately lead to a drop of peel anthocyanin levels in eggplant fruits. The lack or very weak expression of *CHI* in 'L131' may be a consequence of the modulations of the pathway that ultimately lead to the white peel phenotype of this line. Furthermore, the dramatic increase, at the stage C2, of the transcripts level of *SmelGT_ch01*, in all the eggplant lines, as well as *SmelGT_ch05* in 'L131' supports the hypothesis that the enzymes coded by these genes may catalyze the glucosylation of NAR-CHA (**Figures 4** and **7**). The high protein similarity between *SmelGT_ch01* and *SmelGT_ch05* (**Supplementary Data 2**) suggests they could account for a similar function in glycosylating NAR-CHA in the different genetic backgrounds considered (Togami *et al*., 2011).

Coherently with the detected increase of FLAV and CHALC and the simultaneous decrement of ATH compounds at physiological ripening (**Tables 1** and **3**), a concomitant significant reduction of *DFR* transcript in the pigmented lines and boost of that for *FLS* in all the lines were evidenced (**Figure 7**). These metabolic modulations, leading to the switch in phenolic compound composition, may also contribute to explain the remarkable peel coloration changes across the eggplant ripening stages characterized by the appearance of brown/dark-yellow tones in physiologically ripe fruit. *FLS* showed a significant increase of its transcript levels at the physiologically ripe stage C2 when compared to the unripe stages A and B in all the three lines analyzed. *FLS* belongs to the 2-oxoglutarate-dependent dioxygenase family, it is involved in the conversion pathway of NAR to flavonols via flavanone 3- hydroxylase (Kim *et al*., 2010) and it competes with *DFR*, a key step in ATH biosynthesis (Heller and Forkmann, 1988). In the present work, the main derived product of the enzyme coded by *FLS* is likely the compound identified as F1, a glycosyl derivative of kaempferol (**Figure 4**). Together with NAR-CHA, the main phenolic compound at physiological ripe is N2, identified as a glycoside of NAR, most probably the 7-*O*-glucoside. This compound (**Figure 4**) can be formed by GT activities not completely specific for the 2' glycosylation of NAR-CHA alone, yielding both a direct glycosylation of NAR in 7-*O* position (Ogata *et al*., 2004), and glycosylated chalcones in other positions, such as 4' (Togami *et al*.,

2011) followed by a non-enzymatic cyclization, as already evidenced on the equilibrium between naringin and its chalcone (González *et al*., 2002).

We therefore putatively identified a group of genes, represented by two *Tetrahydroxychalcone-2'-glucosyltransferase* (*SmelGT_ch01* and *SmelGT_ch05*) and the *Flavonol synthase* (*SmFLS_ch04),* which show a trend of expression fairly correlated with the variation in the type and content of the compounds detected through biochemical investigation of the eggplant berry development process. The combination of the bibliographic data describing the activity of enzymes orthologous to those encoded by our candidates along with the newly identified phenols is suggestive of their involvement in modulating the evolution of the chemical make-up of eggplant peel during ripening. However, further studies are needed to identify all the genes and regulatory molecular mechanisms leading to the formation of the colorless and pigmented phenols identified in this work, as well as in genotypes displaying different pigmentation (e.g., green or striped).

The peel of physiological ripe eggplant did not show an outstanding decrease of antioxidant capacity with respect to the previous stages of development (**Figure 8**), despite the dramatic changes in its composition. Peroxyl and Fremy's salt scavenging capacities gave trends similar to those reported in previous results by Mennella *et al*. (2012), on hydroxyl and superoxide radical scavenging, respectively. As a consequence, it resulted evident that, during development, anthocyanins, responsible for the peel colour and chlorogenic acid, the main phenols in unripe fruits, are decreased by 75 and 61%, respectively (Table 1), and substituted by flavonoids and chalcones that increased of 120 and 643%, respectively (Table 3), from unripe to fully ripe eggplants. This led to an average slight decrease in total phenols concentration (16 %, Table 1), in a full agreement with antioxidant data.

Comparing the outcomes of the present work with the data of Di Sotto *et al*. (2018), where an experiment of *Herpes simplex* virus (HSV-1) inhibition revealed that only extracts from peels of fruits at physiological ripeness were active, we may suggest that the compounds we identified in relevant amount specifically at the physiologically ripe stage, i. e. NAR-CHA and naringenin 7-*O*-glucoside, could be involved in the HSV-1 inhibition. These compounds are well known as anti-inflammatory and anti-viral agents both on HSV-1 virus both on humans (Hirai *et al*., 2007; Zandi *et al*., 2011) and also on plants (Diaz-Telas *et al*., 2016). Unmarketable physiologically ripe eggplant fruits, currently considered field waste because unsuitable for human consumption (Mauro *et al*., 2020), may therefore be efficiently exploited in nutraceutical and pharmacological sectors as well.

In conclusion, the present work revealed that the accumulation of NAR-CHA, naringenin 7-*O*-glucoside and other flavanones and flavonols characterize the stage of physiologically ripe fruits in *S. melongena*. The genes *SmelFLS_ch04, SmelGT_ch01 and SmelGT_ch05* were identified as strong candidates underlying the switch between the two branches of the phenol biosynthesis pathway leading to a channeling towards NAR-CHA and its glycosylated forms, which occurs during the transition from unripe to ripe eggplant

berries. Although further studies will be necessary for a better understanding of the biosynthesis and accumulation of these phenylpropanoids, the findings reported here are valuable in addressing future breeding goals for the qualitative improvement of eggplant fruits and may therefore have an important practical impact on the cultivation of this crop.

**ABBREVIATIONS USED**

ATH, anthocyanins. CGA, chlorogenic acid. CHA, other chalcones. CHALC, total chalcones. CHI, Chalcone isomerase. CHS, Chalcone synthase. D3R, delphinidin-3-rutinoside. DFR, Dihydroflavonol-4-reductase. EPR, Electron Paramagnetic Resonance. F, flavonol-type compounds. FLAV, total flavanones and flavonols. FLS, Flavonol synthase. GT, glucosyltransferase. N, naringenin derivatives. N2, naringenin 7-*O*-glucoside. NAR, naringenin. NAR-CHA, naringenin chalcone. NAS, nasunin. $ROO^{\bullet}$, peroxyl radical. TP, total polyphenols.

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**Table 1.** Phenol composition (mg/100g dw) of eggplant peels of the four eggplant genotypes 'L131', '305E40', 'HF1' and '67/3' at the four developmental stages A (unripe), B (unripe, commercially mature), C1 (ripe) and C2 (fully ripe).[a]

| | A | | B | | C1 | | C2 | | Average | | % C2/B [b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | mean | ± se | mean | ± se | mean | ± se | mean | ± se | | | |
| **TP** | | | | | | | | | | | |
| L131 | 841.0 | 121.7 | 942.6 | 94.7 | nt | - | 1011.0 | 41.1 | **931.5** | **c** | **7** |
| 305E40 | 2047.4 | 229.9 | 1481.7 | 447.2 | 889.5 | 101.7 | 1100.9 | 195.0 | **1379.9** | **ab** | **-26** |
| HF1 | 1284.7 | 240.1 | 1108.4 | 274.0 | 485.9 | 63.6 | 1110.1 | 96.8 | **997.3** | **bc** | **0** |
| 67-3 | 1876.7 | 131.2 | 1779.2 | 436.0 | 1530.8 | 79.2 | 1257.4 | 254.3 | **1611.0** | **a** | **-29** |
| Average | **1512.5 a** | | **1328.0 ab** | | **968.7 b** | | **1119.9 ab** | | | | **-16** |
| **CGA** | | | | | | | | | | | |
| L131 | 587.4 | 8.7 | 593.5 | 182.5 | nt | - | 164.9 | 14.2 | **448.6** | **b** | **-72** |
| 305E40 | 566.6 | 32.0 | 318.0 | 43.3 | 226.9 | 6.6 | 247.1 | 9.2 | **339.6** | **b** | **-22** |
| HF1 | 534.2 | 100.1 | 500.4 | 80.4 | 110.1 | 21.0 | 230.5 | 13.5 | **343.8** | **b** | **-54** |
| 67-3 | 969.9 | 49.6 | 1087.0 | 253.8 | 854.9 | 33.8 | 343.5 | 26.5 | **813.8** | **a** | **-68** |
| Average | **664.6 a** | | **624.7 a** | | **397.3 b** | | **246.5 b** | | | | **-61** |
| **ATH** | | | | | | | | | | | |
| 305E40 (D3R) | 1088.7 | 181.1 | 943.8 | 371.8 | 267.3 | 89.4 | 169.7 | 19.2 | **617.4** | **a** | **-82** |
| HF1 (NAS) | 488.3 | 71.4 | 324.0 | 129.6 | 87.7 | 38.7 | 104.8 | 24.6 | **251.2** | **b** | **-68** |
| 67-3 (NAS) | 660.3 | 79.5 | 378.5 | 156.9 | 323.4 | 87.5 | 144.9 | 87.5 | **376.8** | **b** | **-62** |
| Average | **745.7 a** | | **548.8 a** | | **226.1 b** | | **139.8 b** | | | | **-75** |

[a] TP, total phenols; CGA, chlorogenic acid; ATH, anthocyanin: delphinidin-3-rutinoside (D3R) in '305E40', or nasunin (NAS) in '67/3' and HF1. Each value is the mean of three biological replications. Within each compound, for Average row and for Average column, different letters indicate significant difference ($P \leq 0.05$) according to Tukey's test. se, standard error. nt, not tested.

[b] Percentage increase of the C2 over the B value.

**Table 2.** Tentative identification of phenol compounds in eggplant peel at C2 (fully ripe) developmental stage by HPLC-DAD-ESI-MS.[a]

| Nr | Code | Rt (min) | $[M + H]^+$/ $[M - H]^-$ | UV $\lambda_{max}$ (nm) | $MS^2$ (m/z) (%) | Compound |
|---|---|---|---|---|---|---|
| 1 | CGA | 15.7 | 355/353 | 239, 292, 324 | 353(100); 191(31) | chlorogenic acid |
| 2 | F1 | 25.5 | 449/447 | 355, 274 | 449(15); 287(100) | kaempferol glycoside |
| 3 | N1 | 21.7 | 597/595 | 279, 304 | 597 (100); 435 (96) | naringenin diglycoside |
| 4 | N2 | 26.0 | 435/433 | 278, 305 | 433(100); 271(45) | naringenin 7-*O*-glucoside (NMR) |
| 5 | - | 27.0 | 535/533 | 355, 274 | 535(100); 287(81) | kaempferol glycoside malonyl ester |
| 6 | CHA2 | 27.9 | 435/433 | 378, 303 | 435(10); 273(100) | naringenin chalcone glucoside |
| 7 | CHA3 | 28.2 | 435/433 | 378/303 | 273(100); 435(25) | naringenin chalcone glucoside |
| 8 | - | 30.1 | 289/288 | - | 289(100); 163(34) | dihydro kaempferol |
| 9 | NAR-CHA | 32.3 | 273/271 | 378/303 | 271(100); 151(10) | naringenin chalcone (NMR) |

[a] Compounds marked by code were those identified in HPLC-DAD separation. Compounds 5 and 8, without a code, were identified only by LC-ESI-MS and not quantified. The structure of compounds 4 and 9 was confirmed, after purification, by NMR and ESI-MS spectroscopy.

**Table 3.** Composition (mg/100g dw) in total flavanones and flavonols (FLAV) and total chalcones (CHALC) of eggplant peels of the four genotypes 'L131', '305E40', 'HF1' and '67/3' at the four developmental stages A (unripe), B (commercially mature), C1 (ripe) and C2 (fully ripe).[a]

| | A | | B | | C1 | | C2 | | Average | | % C2/B [b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | mean | ± se | mean | ± se | mean | ± se | mean | ± se | | | |
| **FLAV** | | | | | | | | | | | |
| L131 | 220.8 | 87.0 | 321.4 | 189.5 | nt | - | 549.8 | 106.6 | **364.0** | **a** | **71** |
| 305E40 | 362.9 | 31.3 | 203.5 | 58.2 | 329.3 | 28.6 | 528.5 | 194.1 | **356.1** | **a** | **160** |
| HF1 | 215.9 | 54.4 | 256.1 | 70.4 | 238.3 | 22.4 | 578.7 | 106.3 | **322.3** | **a** | **126** |
| 67-3 | 207.9 | 31.3 | 289.7 | 58.2 | 331.6 | 28.6 | 703.7 | 20.8 | **383.2** | **a** | **143** |
| Average | **251.9** | **b** | **267.7** | **b** | **299.8** | **b** | **590.2** | **a** | | | **120** |
| **CHALC** | | | | | | | | | | | |
| L131 | 32.8 | 15.5 | 27.7 | 11.9 | nt | - | 296.2 | 48.6 | **118.9** | **a** | **970** |
| 305E40 | 29.3 | 6.5 | 16.4 | 2.7 | 66.0 | 37.6 | 155.6 | 25.0 | **66.8** | **bc** | **849** |
| HF1 | 46.3 | 29.9 | 27.9 | 7.8 | 9.8 | 20.0 | 196.0 | 10.5 | **80.0** | **b** | **603** |
| 67-3 | 38.7 | 12.2 | 24.1 | 11.5 | 20.7 | 6.4 | 65.4 | 0.5 | **37.2** | **c** | **171** |
| Average | **36.8** | **c** | **24.0** | **c** | **45.5** | **b** | **178.3** | **a** | | | **642** |

[a] Each value is the mean of three biological replications. Within each compound, for Average row and for Average column, different letters indicate significant difference ($P \leq 0.05$) according to Tukey's test. se, standard error. nt, not tested

[b] Percentage increase of the C2 over the B value.

**FIGURE CAPTIONS**

**Figure 1.** Fruits at the four stages of development (A, unripe; B, commercially mature; C1, ripe and C2, fully ripe) in eggplant lines characterized by lilac ('67/3'), dark purple ('305E40') and white ('L131') peel color.

**Figure 2.** HPLC-RP-DAD chromatogram of semi-purified extract of eggplant peel from four pooled genotypes at C2 fully ripe stage. The top plot is registered at 280 nm, the bottom one at 355 nm. The main tentatively identified compounds at 280 nm were indicated as chlorogenic acid (CGA), naringenin derivatives (N1 and N2) and naringenin (NAR). The compounds tentatively identified at 355 nm were indicated as chlorogenic acid (CGA), naringenin chalcone derivatives (CHA1, CHA2 and CHA3), a flavonol type compound (F1) and naringenin chalcone (NAR-CHA).

**Figure 3.** HPLC chromatograms of four pooled genotypes of eggplant semi-purified extract at fully ripe stage C2 registered at 280 nm (top) and 355 nm (bottom) with the compounds identified by LC-ESI-MS. Each peak is identified by retention time and code (see Table 2 for the list of compounds and Materials and Methods for the analytical parameters).

**Figure 4.** Scheme of flavonoid synthesis in eggplant peel. The identified genes putatively encoding keys enzyme were reported. Genes resulted statistically up regulated at the stage C2 with respect to the stage A and B in the lines '67/3', '305E40' and 'L131' have been marked (*), those down regulated are marked (#).

**Figure 5.** Average values of 4 eggplant genotypes of flavonol-derivative (F1), naringenin derivative (N2), naringenin chalcone (NAR-CHA) and naringenin (NAR) in peels at four different developmental stages (A, unripe; B, commercially mature; C1, ripe and C2, fully ripe). The error bars represent the values of standard error, and different letters are for significant differences within the same class of compounds ($P \leq 0.05$).

**Figure 6.** Evolutionary relationships among eggplant GT proteins of interest (SMEL). The tree was obtained using peptide sequences from carnation (Dic), cyclamen (Cy), and catharanthus (Cr) reported in the literature (Ogata et al., 2004; Togami et al., 2011) to be involved in the formation of chalcone 2'-O glucoside (blue dots) and eggplant sequences obtained by homology search. The sequence for a petunia 5GT anthocyanin 5-O-glucosyltransferase used also in Togami et al. (2011) was included as outgroup. Red arrowheads indicate the genes chosen for subsequent RT-qPCR analysis. Only bootstrap values above 50 are shown.

**Figure 7.** RT-qPCR transcription profiling of *SmelCHS_ch00*, *SmelCHS_ch05, SmelCHI_ch10, SmelCHI_ch00, SmelGT_ch01, SmelGT_ch05, SmelDFR_ch00* and *SmelFLS_ch04* in the pigmented eggplant accessions '67/3' (A), '305E40' (B) and 'L131' (C) at three stages of fruit development: unripe (stage A), commercially mature (stage B) and physiologically ripe (stage C2). Expression levels, measured by RT-qPCR, are shown as relative units using *SmelGAPDH* and *Smel18S* as reference gene. Data are means of three biological replicates ± standard deviation. One-way ANOVA with Tukey's HSD post hoc test was performed. Different letters indicate significant differences at $P \leq 0.05$.

**Figure 8.** Average values in eggplant peels of 4 genotypes of antioxidant indexes by peroxyl radical (a) and Fremy's salt (b) scavenging at four different developmental stages (A, unripe; B, commercially mature; C1, ripe and C2, fully ripe). The error bars represent the values of standard error, and different letters are for significant differences within the same class of compounds ($P \leq 0.05$).

**SUPPLEMENTARY DATA**

Supplementary Table 1. Sequences of the primers used to carry out the RT-qPCR.

Supplementary Data 1. CDS of the genes analyzed by RT-qPCR.

Supplementary Data 2. Peptide sequences used for Neighbor-Joining analysis.

Supplementary Figure 1. HPLC profile at 280 nm of fractions B vs the semi-purified extract, with the UV-VIS spectrum of the purified compound N2.

Supplementary Figure 2. HPLC profile at 355 nm of fractions C vs the semi-purified extract, with the UV-VIS spectrum of the purified compound NAR-CHA.

Supplementary Figure 3. HPLC profile at 355 nm of fractions B vs the semi-purified extract, with the UV-VIS spectrum of the purified compounds F1 and CHA3.