Insights in the fruit flesh browning mechanisms in *Solanum melongena* genetic lines with opposite post-cut behavior

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Abstract (100-150 words)

Color, taste, flavor, nutritional value and shelf life are important factors determining quality and healthiness of food and vegetables. These factors are strongly affected by browning processes, occurring after fruit or vegetable cutting. Ten eggplant genetic lines characterized for chlorogenic acid (CGA) content, total phenols (TP), polyphenoloxidase (PPO) activity and browning tendency corroborated a lack of significant correlations between biochemical factors and fruit flesh browning. In depth molecular and biochemical analyses of two divergent eggplant genetic lines, AM199 (high browning) and AM086 (low browning) within 30 minutes from cutting, highlighted differences in the physiological mechanisms underlying the browning process. qRT-PCR analysis revealed distinct activation mechanisms of CGA biosynthetic genes and PPOs expression in the two genetic lines, and metabolic data on CGA, sugars and ascorbic acid contents confirmed that their different browning tendency matched with different metabolic responses to cutting. Our findings suggest that the complex mechanism of flesh browning in the two eggplant genetic lines might be mediated by multiple specific factors.

Keywords: Solanaceae, eggplant, browning, antioxidants, qRT-PCR

Introduction

Enzymatic browning is a color reaction occurring in cut fruits and vegetables such as apple, pear, banana, peach, lettuce, potato and eggplant, which are highly susceptible to enzymatic oxidation during postharvest processing and storage. Extensive browning of cut fruit and vegetable surface compromises quality maintenance and usually impairs the properties of the product as it negatively effects on aesthetic appearance ,taste and texture, as well as reducing nutritional value[1]., For all these reasons, this deteriorative reaction is a major economic problem both for ready-to-eat fresh-cut fruit and vegetable producers as well as for consumers. Polyphenoloxidase (PPO) enzymes and antioxidant phenolic compounds are reported to have a major role in browning processes[2]. Knife cutting determines damage to fruits or vegetables membrane integrity by cellular disruption, which favors the release of phenolic compounds from the vacuole, PPOs from the plastids and oxygen penetration. Enzymatic browning mediated by PPOs is a two step reaction consisting, firstly, in the enzymatic oxidation of monophenols or *o*-diphenols to yield *o*-quinones, followed by condensation or polymerization reactions[3]. Given the high number of monophenolic and/or diphenolic compounds present in plant tissues, the interaction between PPO-and phenolic substrates may form a variety of colored quinones[4,5]. However, dark brown coloration is mostly due to the non enzymatic formation of *o*-quinones complex polymers, known as melanins[2,6]. Quinones are highly reactive intermediate compounds that readily polymerize and by reacting with the nucleophilic side chain of amino acids, they are also able to crosslink proteins, thus affecting their availability. It's well known that quinones and their complex melanins are involved either in reducing pathogens amino acid assimilation and in increasing the cell wall resistance to insects and pathogens[7];consequently, PPOs are the major players against herbivores attack [7].

The common natural substrates of PPOs are usually phenolics, which are often species-specific, such as dopamine in banana[8], catechin in grape, tea and apple[9],orchlorogenic acid in apple and eggplant[10]. Therefore, different types of PPOs might have specific enzyme affinities for different phenolics, and accordingly to this hypothesis their combined interactions might result in a different degree of browning. To prevent the browning negative effects on food processing, several inhibitory strategies relying on chemical additives have been proposed. Many of them are based either on the use of benzoic acids and their derivatives, which compete for the natural substrate or on the application of ascorbic acid , citric acid and ossalic acid , which block the PPO enzymes by chelating the copper ion in the active site [11,12]. However, it has been shown that the use of these inhibitory agents may compromise food safety[13]. Therefore, alternative strategies have been developed in several species also including the use of genetic engineering. Silencing strategies of the PPOs gene family, also including micro RNA[14], were proved to be effective in a reduction of the browning process, albeit possible side effects on plant resistance to biotic and abiotic stresses need to be further ascertained. For example, in tomato fruits, PPOs silencing, resulted in a reduced browning capacity, although an increased plant susceptibility to infectious diseases has been reported[15–18]. Similarly, walnut PPOs RNAi transgenic lines showed a reduced cathecol oxidase activity but developed necrotic lesions on the leaves during plant growth[19].

Eggplant (*Solanum melongena* L.) is ranked amongst the top ten vegetables (and the first among Solanaceous crops) in term of oxygen radical absorbance capacity (ORAC) due to the high accumulation of phenolics constituents [20]mainly composed by chlorogenic acid[21] in fruits. The high affinity of PPOs for chlorogenic acid[22] suggests that these two components are the causal factors in post cutting enzymatic browning processes in eggplant. In order to help selective breeding of eggplant lines with high CGA content and low browning, an interspecific population between *S. melongena* and *S. incanum* was developed[23], which allowed mapping of both the PPOs and the CGA biosynthetic genes. The CGA biosynthetic genes were mapped on five different linkage groups, allowing to an ease selection for higher CGA content, whereas PPO genes, by clustering in the same linkage group, made difficult recombinants selection.

Nevertheless, eggplant fruits with high content of CGA and fully-operating oxidizing enzymatic machinery not always undergo to an extensive browning and a significant loss of nutritional value. In several eggplant cultivars[24] as well as in a large collection of potato tubers[25], the lack of a positive correlation between browning, CGA content or PPO activity indicated that additional factors are influencing fruit flesh browning.

In the regard, it’s worth consider that knife cutting is perceived by fruit surface as a wounding process, which is known to trigger a defence response by several changes in the primary or secondary metabolism, therefore also carbohydrates and organic acids might also be influencing browning tendency.

As a further contribution to the understanding of this complex mechanism in eggplant, here we report our biochemical studies on 10 eggplant lines that allowed the selection of two lines characterized by an opposite tendency to browning and then the results of an in-depth investigation of the biochemical and molecular changes occurring in the two selected lines during a post-cutting time course.

Materials and Methods

Plant material and cultivation

The ten eggplant genetic lines used in this study belong to a collection of breeding lines of CREA-ORL, namely 1F$_5$(9) (oval dark purple), CCR3 (long pale purple), 305E40 (long dark purple), 67/3, (round violet), Tal1/1 (long dark purple), and of local varieties 553 Palermitana (Italy, round violet), AM086 (Japan, LS3805 Minden, round, greenish violet), AM199 (Thailand, TH470, ribbed round pink) and AM167 Angio (China, long dark violet), along with HF$_1$ 305E40x67/3, the F$_1$ hybrid of a mapping intraspecific population as previously reported by [26]. Plants were cultivated during the summer of 2013 in the experimental farm of the Consiglio per la ricerca in agricoltura e l’analisi dell'economia agraria, Centro di Ricerca per l'Orticoltura (CREA-ORT) in Battipaglia (Italy). Plants were transplanted to field according to a randomized block design with three replications each composed by six plants. For each replication, 5-8 fruits harvested from different plants at commercially ripe stage (stage B, approximately 38 days after flowering), visually evaluated according to skin color and absence of mature seeds, were collected. The experimental samples, constituted by portion of all the fruits collected after browning evaluation were immediately frozen in liquid N_2 and stored at -80°C or in part lyophilized until further biochemical analyses

Evaluation of the fruit browning

For evaluation of browning tendency, several fruits (4 or more) from the ten eggplant lines were measured and used for obtaining a mean value for each replication. Fruits were cleaned in potable water and peeled. Flesh slices of about 2 cm height, transversally cut at the midpoint between the blossom and stem ends, were exposed to air for 30 min after cutting. A well-sharpened knife with a straight edge was used to produce clean cuts. Dark color development was both visually scored using an arbitrary scale ranging from 0 (no variation between 0 min and 30 min) to 4 (high browning at T30) and determined with a Minolta chroma-meter (CR-300; Minolta Co. Ltd., Osaka, Japan), fitted with an 8-mm-diameter aperture and expressed in the "CIELAB 1976 color coordinates. Measurements were made in the central part of the fruit,, avoiding both the seed locules and the flesh next to the peel region, immediately after cutting (T0) and 30 min later (T30). The whiteness of the fruit flesh was measured as the Euclidean distance of the color coordinates to the pure white color coordinates ($L^* = 100$, $a^* = 0$, $b^* = 0$) using the formula $DW=[(100-L^*)^2+a^{*2}+b^{*2})]^{0,5}$ where DW is the distance to the pure white color and L*(Lightness variable), a* and b* (Chromaticity coordinates) is the uniform color space defined by the CIELAB. The difference between DW at 30 min (DW_{30}) and at 0 min (DW_0) after the fruit was cut was used as a measure of degree of browning (DB) suffered by the fruit (degree of browning = $DW_{30} – DW_0$) as it should correspond to an increase in the distance to pure white. The color difference was measured as the Euclidean distance between the color coordinates at 0 and 030 min after the cut CD=$[(L^*_{30}-L^*_0)^2+(a^*_{30}-a^*_0)^2+(b^*_{30}-b^*_0)^2]^{0,5}$. Both measures give different and complementary information related to the evolution of color.

Biochemical characterization of eggplant genetic lines

Fruit slices were collected at T0 and freeze-lyophilized for biochemical analyses, namely CGA content, total phenols quantification and polyphenoloxidase activity. CGA was extracted and analyzed according to[21] with minor modifications. The analyses were performed with a Waters E-Alliance HPLC system constituted by a 2695 separations module with quaternary pump, autosampler, and a 2996 photodiodearray detector. Data were acquired and analyzed with Waters Empower software. A binary mobile phase gradient of methanol in 0.01% aqueous phosphoric acid was used according to this procedure: 0-15 min, linear increase from 5 to 25% methanol; 15-28 min, linear increase from 25 to 50% methanol; 28-30 min, linear increase from 50 to 100% methanol; 30-32min, 100%methanol; 32-36 min, linear decrease from 100 to 5% methanol; 36-43 min, 5% methanol. The flow rate was 0.8 mL/min. Quantification of CGA, carried out after RP-HPLC separation, was based on absorbance at 325 nm relative to the sesamol internal standard and an external standard of authentic chlorogenic acid (Sigma-Aldrich, St. Louis, MO). The results were expressed as µmol/100 g of dry weight (dw).

Total phenols (TP) and PPO activity were assayed through spectrophotometric methods. TP were evaluated by a modified Folin-Ciocalteu method [27]of a McIlvaine buffer (pH 3.0, 1mL) extract of 30 mg of lyophilized tissue. Results were expressed as µmol of CGA equivalent per 100 g of dw. Chlorogenic acid was used because it is the main phenolic compound in the eggplant fruit. The PPO activity was assayed following the Fujita and Tono method,[28] using 30 mg of lyophilized fruit extracted with 1 mL of McIlvaine buffer (pH 5.0). Results were expressed as U/100 mg of dw, with 1U=0.01 absorbance unit variation/min, using chlorogenic acid as the substrate at 420 nm.

Characterization of secondary metabolites in AM199 and AM086 genetic lines used for post cut browning experiments were performed by LC-MS analysis

Secondary metabolites were extracted from the fruit flesh of AM199 and AM086 according to the following protocol.

Briefly, 5 mg of lyophilized samples were extracted in 1.5mL of 75% (v/v) methanol containing 0.05% (v/v) trifluoroacetic acid (TFA). After homogenization, the samples were stirred for 40 min and centrifuged at 19,000 x *g* for 10min. The extracts were filtered through 0.2 µm polytetrafluoroethylene (PTFE) filters. For each line, three biological replicates were prepared. Each biological replicate was analyzed in triplicate. All the extracts were analyzed using reversed phase liquid chromatography coupled to a photodiode array detector and to an ion trap mass spectrometry (LC–PDA–MS) system. Such a system consisted of an ultra-performance liquid chromatography (UPLC) DIONEX Ultimate 3000 model coupled to an LTQ XL mass spectrometer (Thermofisher Scientific, Sunnyvale, CA, USA). A 5 µL aliquot of sample was injected on a LunaC18 (100x2.0mm, 2.5µm particle size) column equipped with a Security Guard column (3.0x4.0mm) from Phenomenex. The separations were carried out using a binary gradient of ultrapure water (A) and acetonitrile (B), both acidified with 0.1% (v/v) formic acid, with a flow rate of 0.22 mL min^{-1}. The initial solvent composition consisted of 95% (v/v) of A and 5% (v/v) of B; increased linearly to 25% A and 75% B in 25min and maintained for 1min; returned to 95% of A in 1min. The column was equilibrated to 95% A and 5% B for 11min before the next injection. The analysis lasted for 38min and the column temperature was set to 40° C. Mass spectra were obtained in positive ion mode over the range *m/z* 70–1,400. The capillary voltages were set at 9.95V and the source temperature was 34° C. The putative identification of some metabolites was confirmed by *m/z* data from authentic, distinct standards such as shikimic acid and quinic acid (purchased from Sigma-Aldrich), solamargine (SM) and solasonine (SS) (kindly provided by Prof. Adelia Emilia de Almeida). Whereas, dicaffeoyl-spermidine (DCS) and malonyl-solamargine (MSM) were tentatively identified based on Ms/Ms fragments identity and retention time (r. t.) by matching the resulting spectra with those reported in *Solanum melongena* secondary metabolites database. Xcalibur software (ThermoFisher Scientific) was used to control all instruments and for data acquisition and data analysis.

Post cut browning experiments in AM086 and AM199 eggplant genetic lines

For in-depth characterization of the browning process, a time course experiment was conducted on sliced eggplants. On the basis of the opposite browning tendency, two selected eggplant genetic lines AM086 and AM199 in more than 18 plants for each line were grown in pots during the summer of 2014 in a greenhouse located at the Research Unit of the Institute of Biosciences and Bioresources (CNR) in Portici (Italy). For each genetic line, three biological replicates each composed by a minimum number of 6 fruits from 6 six different plants were collected at commercial ripe (as stated above). The fruits were harvested and used for the browning experiment on the same day. The collected fruits were cleaned and peeled, then slices of about 2 cm of thickness, obtained using a commercial vegetable cutting knife (blade thickness about 0.25 mm) were exposed to air. Experimental samples were collected at three time points, zero (T0), fifteen (T1) and thirty (T2) minutes after cutting. For each biological replicate, the slices from different fruits were pooled, frozen in liquid nitrogen and stored or lyophilized for further biochemical and molecular analysis.

qRT-PCR analyses

Total RNA was extracted from 100mg of fruit flesh of both AM086 and AM199 *S. melongena* genetic lines by using RNAsy kit (Quiagen, Valencia, CA, USA.) according to manufacturer's instructions. RNA was quantified with a NanoDrop 2000c spectrophotometer (NanoDrop Technologies, Wilmington, USA).Using a SuperScript™ first-strand cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA), first-strand cDNA was synthesized by reverse transcription (RT) to transcribe poly (A)$^+$mRNA with oligo-dT primers following the manufacturer's instructions.

Fruit flesh-specific expression of phenylpropanoid and PPOs genes was analyzed using RT-qPCR with anABI7900 HT (Life Technologies, Carlsbad, CA, USA). To amplify gene fragments, cDNA templates were used in a 20 µL PCR reaction according to manufacturer's instructions. For PPOs expression profiles the high sequence similarity characterizing the six eggplant PPO isoforms[29] did not allow to design optimal and isoform-specific q-PCR primers; therefore common regions were used to design primer sequences specific for two groups of polyphenoloxidases, PPOA(1 to 3)and PPOB (4 to 6) (Table 4).The PCR reactions were performed as reported by [30]. The analysis was done on three biological replicates and in technical triplicates. Normalization was performed by using adenine phosphoribosyltransferase (APRT) gene as housekeeping gene[31]. Quantitative relative results were expressed in the form of relative expression by using the $^{\Delta\Delta}$Ctmethod, by using the respective T0 sample as internal calibrator. No template control reactions were included on each plate, and each sample was tested for gDNA contamination using a minus RT control. A list of the analyzed genes, accession numbers, and primer sequences can be found in Table 4

Ascorbic acid content

Ascorbic acid (AsA) content was determined from an aqueous extract of lyophilized fruit flesh powder (100mg plus 3mL of 6% metaphosphoricacid in distilled water), homogenized for 30 s through an Ultra-Turrax, and then centrifuged at 3000 rpm for 15 min. The extraction was repeated twice on the pellet, and the supernatant was collected each time and finally made to 10 mL with the extracting solvent. The extracts were filtered through 0.2 µm PTFE filters and analysed at room temperature by the same UPLC system as for the secondary metabolites analyses. A 5 µL sample was injected on a Kinetex (75 x 4.6 mm, 100 Å, particle size 2.6 µm) column (Phenomenex, Torrance, CA, USA). The mobile phase was constituted by a 0.02M H_3PO_4 aqueous solution ata flow rate of 0.35 mL/min. Quantification of AsA was made at 254nm using a calibration curve of the authentic chemical standard of AsA from Sigma-Aldrich.

Sugars analyses

300mg of lyophilized sample were dissolved in deionized water (10mL), 30’’vortexed and sonicated in a water bath for 5 min. After centrifugation 30 min 4°C 12000 rpm, supernatant was 10 times diluted with H_20 and passed through 0.45 µm filter. The extract was injected on a HPLC system, equipped with a PU 880 Jasco pump, a Jasco AS-1555 autosampler, a heating system for chromatographic column Jones Chromatography mod 7971 and a refractive index detector Jasco RI-930.

The chromatographic condition has been as follows: Biorad Carbohydrate Analysis Aminex HPX-87P, 300 × 7.8 mm, kept at 85°C, mobile phase MilliQ 18Ω H_2O warmed at 60°C at a flow of 0.7 mL/min, while the injection volume has been of 40 µL.

Sugars were quantified by calibration curves constructed with commercial standards of sucrose (retention time 7.3 min), glucose (8.7 min) and fructose (10.4 min).

Statistical analyses

Biochemical data were subjected to ANOVA, by JMP software (SAS Institute, Cary, NC, USA), according to a completely randomized design. Means were compared using the Tukey HSD test ($p \leq 0.05$). Pearson product moment correlation coefficients (r values) were calculated using the means of metabolite concentrations, enzymatic activity, total phenolics, colorimetric data and browning index. Moreover, ANOVA statistical analyses and Tukey HSD test ($p \leq 0.05$) were performed on qRT-PCR data for each eggplant line by using SygmaPlot software.

Results

Characterization of eggplant genetic lines

Browning index was determined in the ten eggplant genetic lines both by visual inspection and by using a Minolta chroma-meter (Table 1).Visual evaluation of fruit flesh browning 30 minutes after cutting, was defined by using an arbitrary scale from 0 to 4, corresponding to minimum and maximum browning, respectively. Most eggplant lines showed a browning index between 1.08 and 2.18, with the exceptions of AM199, which showed the highest browning index (3.4), and, conversely, lines AM167 and AM086, which had the lowest tendency to browning (0.50 and 0.46, respectively; Table 1). The values detected by the chroma-meter indicated that DW0 for the fruit flesh ranged from 15.54 for 67/3 to 41.31 for Tal 1/1. For DW30, values ranged from 19.75 for 67/3 to 42.48 in AM199 (Table 1). The unexpected high values of DW0 detected in some cases are due either to the yellowish-greenish fruit color characterizing the flesh of some accessions (affecting either a* and b* values, which have a consistent effect in the calculation of DW_0), or to an immediate browning of the pulp within few seconds from the cutting (it is the case of the genotype AM199, this phenomenon mostly affecting the L*value). The higher values detected for DW_{30} are mostly due to an effective browning tendency of the flesh with respect to T0, which affects in all the genotypes the L* component of the color value (Table 1). However, for some genotypes characterized by greenish flesh, like Tal1/1, the color change from T0 to T30 does not affect only the L* component (the most related to darkening) but also the other two component a* and b* (as the greenish color tends to convert in yellow-brown), which have also a severe effect on the DW calculation. The changes in all components of the color affect the DW_{30} value, therefore in the case of Tal1/1 the difference between DW_{30} and DW_0 has a negative result. DB values, calculated as the differences between DW_{30} and DW_0, range from-2.30 and 8.07. According to [32], these values could be related to an increase in the distance to pure white in samples which suffered the browning processes.

The color difference values (CD), calculated as Euclidean distance between the color coordinates at 0 and 30 min after the cut, range from 2.39 in AM086 to 15.18 for AM199. Both CD and DB measures give different and complementary information related to the evolution of color, however, CD values resulted more significantly correlated with the data obtained from phenotypic evaluation (r = 0.87).

Both methodologies (phenotypic and measured through CD) for the evaluation of browning index allowed to detect a considerable variation for browning tendency between the genetic lines, however AM199 as well as AM086 and AM167 were well distinguishable for their opposite browning tendency.

Both HPLC and LC-MS analyses of methanolic extracts from the flesh sliced fruits revealed that, in all the eggplant lines, CGA was the main metabolite detected at 325 nm. As shown in Table 2 CGA varied from a minimum of 0.7mg/100mg dw for AM167 to a maximum of 2.0mg /100mg dw for AM199. Being CGA the main phenolic compound detected in the extracts (Supplementary figure X Chromatogramma), it was used as reference for the determination of total phenols by the Folin Ciocalteu method. Paralleling the trend observed for CGA, total phenolics measured in lines AM167 and AM199 were 2.2±0.5 and 4.6±0.2mg CGA eq. /100 mgdw, respectively, in agreement with the high positive Pearson moment correlation ($r=0.94$ $p<0.0001$) found between CGA and total phenolics. Chlorogenic acid content in the fruit flesh was on average 36.1% of the total phenols, ranging from 29.9% in the 305E40 inbreed line to 44.0% in AM199.
The measurement of PPO activity revealed thatTAL1/1 had the highest activity, whilea decreased order was detected in the other eggplant lines CCR3>1F$_5$(9)>AM199>AM167>305E40. Beside 67/3, which had the lowest PPO activity, the remaining lines AM086, Palermitana, and HF$_1$ 305E40x67/3 showed a comparably low PPO activity, which was almost halved as compared to CCR3. In our ten lines the PPO activity correlated neither with CGA nor with total phenolics ($r= -0.00190$ $p>0.05$, and $r= -0.0581$, respectively, $p>0.05$). Interestingly, the tendency to browning was not significantly correlated with any of the other parameters ($p>0.05$).

Molecular changes associated with browning

Since we found no correlation between the browning index and polyphenoloxidase activity or CGA content in the fruit flesh (Table 2), the two eggplant lines with opposite tendency to browning, i.e. AM199 and AM086, were chosen to further investigate the post-cutting browning process during a time course experiment with three time points, immediately after cutting (T0), 15 min (T1) and 30 min post cutting. As expected, the sliced AM086 fruit flesh did not show an intense oxidation during the duration of the time course, with only seeds turning dark yellow, while a marked browning occurred inAM199 sliced fruits already few minutes post cutting and in 15 minutes, which is not limited to the seed surrounding area, but affected most of the flesh surface (Figure 1a).

The molecular cues possibly associated to the different post-cut behavior of the two lines were investigated during the time-course experiment through transcriptional analysis of putative major players of the browning process[33,34], namely genes involved in CGA biosynthesis and in the enzymatic oxidation mediated by polyphenoloxidases (Figure 1b and 2). The genes encoding for the CGA biosynthetic enzymes phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxilase (C4H), coumarate ligase (4CL), hydroxycinnamoylquinate-transferase (HQT) and cinnamate 3'-hydroxilase (C3H) showed expression profiles indicating a distinct pattern of gene activation during the time course for the two eggplant lines AM086 and AM199 (Figure2). Overall, the abundance of almost all the analyzed CGA biosynthetic gene transcripts was higher in AM086 than in AM199. During the time course, *PAL*, *4CL*, *C4H*, *HQT* and *C3H* showed a gradual induction from T0 to T2 in AM086, with the only exception of 4CL at T2. Noticeably, this transcriptional increase was particularly relevant for *PAL* and *HQT*. Interestingly, CGA biosynthetic genes in AM199 showed an almost opposite trend, with an unchanging or decreasing transcriptional activity observed for all genes, besides *HQT*, for which a slight increase of expression from T0 to T1 was detected.

Transcriptional analysis was also performed on the polyphenoloxidase genes, using primers designed to amplify group A (PPOs 1 to 3) and group B (PPOs from 4 to 6) genes (Figure 2).A distinct temporal activation was observed between the two lines, since the expression of the two polyphenoloxidase groups significantly increased from time T0 to T2 in AM086, whereas in AM199 a strong transcript induction was detected only for the PPO group A 15 min after cutting (T1), followed by a rapid decline at 30 min (T2).

Since PPOs are known to participate in the late plant response to wounding, we measured the expression levels of two other major players of this response, namely, proteinase inhibitor genes I and II (PIN I and II). In AM086 both PIN I and II did not show any significant induction, whereas in AM199 a one-fold induction of PINII was already detected 15 minutes upon cutting, which was maintained at T2 (Figure 3).

This data, along with the strong induction of PPOs A in AM199, supported the idea that a different post-cut signaling response is occurring in the two lines.

Biochemical changes associated with browning

Biochemical characterization of possible players of the browning process was also conducted in AM199 and AM086 eggplant lines during the post-cut time course (T0, T1 and T2).

The differences in CGA accumulation observed in field-grown fruits between AM199 and AM086 were also confirmed in greenhouse-grown fruits, with a double amount of CGA in AM199 relative to AM086 (Fig. 4A, T0). HPLC analyses revealed that, after cutting, AM086 sliced fruits had a significant increase of CGA, from about 1 mg/100mg dw at T0 to 2 mg/100 mg dw at T1 and T2, whereas no significant changes were observed during the time course in the highly-browning line AM199 (Fig. 5A).

An approximately halved amount of quinic acid was already detected in AM086 fifteen minutes after cutting, whereas in AM199 a significant reduction was observed at time T2. A three times reduction of shikimic acid was also detected at T2 for AM086, while a non-significant reduction of shikimic acid was found in AM199 (Table 3).

Glycoalkaloids pattern of accumulation also differed between the two genetic lines. A higher basal content of glycoalkaloids was detected in AM199, where at least a double amount of solasonine (SS) and solamargine (SM), and a four times higher content of methylsolamargine (MSM) were measured than in AM086.In AM199 a gradual increase was evidenced between T0 and T2, which was significant for SS and SM and not for MSM, whereas a decrease between T0 and T1and an increase between T1 and T2 characterized for the same compounds AM086 (Table 3).

In order to investigate whether other antioxidants rather than CGA could interfere in the oxidation process we evaluated ascorbic acid (AsA) accumulation in the two genetic lines over the time course. AM86 and AM199 showed comparable levels of AsA at time T0. In addition, AsA accumulation did not significantly vary during the time course (Table 3).

HPLC analysis of sugar composition revealed that the amount of glucose, fructose and sucrose in AM086 was approximately two times higher than in AM199 at T0 (Fig. 4B). Interestingly, AM086 showed a significant reduction of the content of glucose, fructose and sucrose during the time course, while the content of the three sugars remained almost constant in AM199 (Fig 4B).

Discussion

Post cut browning is not only dependant on phenolics and polyphenoloxidase activity

Browning of fresh cut fruits and vegetables has strong detrimental effects on processed food appearance and taste, and consequently results in large economic losses for fourth-range products sellers. Since enzymatic browning is initiated by PPOs-mediated oxidation of phenols, PPO activity, phenolic concentration, pH, temperature and available $O_{2,}$ have generally been considered the major determining factors of the browningrate[2]. The lack of positive relationships between nutritional traits, namely accumulation of antioxidant compounds, PPO activity and browning has been already reported[21,24,32–34] thus suggesting that other multiple causes might affect the browning process[35,36]. For this reason, we investigated whether additional factors might possibly play a role in the browning process of eggplant fruit flesh. Firstly, we evaluated a collection of ten genetically diverse Occidental and Oriental eggplant genetic lines, belonging to different morphology-based group[30],for the widely recognized candidate determinants of browning, i.e. CGA and total phenols content, and PPO activity, along with browning tendency. Albeit the number of eggplant genetic lines used was limited, the wide detected variation of the biochemical parameters was comparable to the variation observed within larger collections[24,37]. Consistently with previous studies[32,33,38], we found a positive correlation between CGA and total phenols in the analyzed genetic lines, but a lack of correlation between flesh browning and phenols content or PPO activity, as shown by the TAL1/1 line, which had the highest PPO activity and a high CGA content, but did not show severe post cut browning (Table 1-2). Besides, no correlation was detected between the browning behavior and fruit morphology or geographical origin of the genetic lines

Cutting induces distinct metabolic and molecular changes in fruits of AM199 and AM086

The two Oriental linesAM199 and AM086 were then selected for further investigations in a post cutting time course experiment on the basis of their opposite tendency to browning (Table 1 and Figure 1).

A distinct temporal activation of gene expression was observed between the two genetic lines during the time course. CGA biosynthetic genes showed a fast transcriptional activation in AM086 within fifteen minutes after cutting, while they were almost switched off inAM199 after thirty minutes. Notably, in AM086 sliced fruits, the trend of transcript accumulation for the two key CGA biosynthetic genes, PAL and HQT, paralleled CGA accumulation (Fig. 1b and Fig. 3). In addition, the same line displayed a strong reduction of quinic acid, as well as depletion of shikimic acid (at T2) during the time course (Table 3), suggesting an active fueling of new quinic acid and chorismate for CGA formation and for providing phenylalanine into the general phenylpropanoid pathway, respectively. On the contrary, the decreased transcriptional activity of CGA biosynthetic genes in AM199 during the time course is consistently associated with the lack of significant changes in CGA content (Table 3). Thirty minutes post cutting, a reduction of quinic acid was also observed, whereas no significant variation in shikimic acid content was detected, thus indicating that either slow anabolic processes were ongoing or that quinic acid might be used as substrate for oxidation[39,40]

PPO genes were expressed in intact fruits of both eggplant genetic lines, but displayed a different post-cut induction pattern (Fig 2). A strong and progressively increased expression of both PPO A and B was detected in AM086, which paralleled the transcriptional trend observed for CGA biosynthetic genes, whereas in AM199 only PPO A transcripts showed a strong and transient accumulation fifteen minutes post cutting. It has been reported that substrate specificity of PPOs can vary between species and even between cultivars[3]. Therefore it might be argued that the different browning tendency between AM086 and AM199 might be the result of the reported distinct PPOs substrate affinity[3]. The absence of browning in AM086 could be associated either to a lower availability of appropriate substrates for PPOs or to the inactivity of PPOs towards phenolic substrates[41,42].

Since the browning tendency might be influenced by the concentration of reducing agents, we measured ascorbic acid content in AM086 and AM199, which was very low and did not significantly differ both between the two lines and between different time points during the time course (Table 3). Therefore prevention of melanin formation by ascorbic acid[43,44] does not account for the different browning tendency of AM086 and AM199.

Upon cutting, a significant reduction of sucrose, fructose and glucose occurred in AM086, while no significant changes were detected in AM199. We might speculate that, in AM086, the catabolism of sucrose contributes to provide fructose and glucose to be readily used for glycolysis and cell wall formation[45]. Indeed, we cannot exclude that the strong reduction of sugars in AM086 might serve for glycosylation of phenolics, thus influencing PPO activity and blocking browning processes. By contrast, in AM199, it cannot be assumed that browning is the result of carbohydrate oxidation, or of a non enzymatic formation of glucose-fructose-amino acid systems[46–48].

Taken together, the marked differences in post-cut changes that we detected between the two eggplant genetic lines could be the consequence of a different sensing and/or response to the damage caused by mechanical injury.

Flesh browning can be part of a defensive strategy

In AM086 the cutting-triggered cascade of molecular and metabolic events resembles a typical defense response to wounding. This includes transcriptional activation of the phenylpropanoid metabolism and the accumulation of hydroxycinnamic acids (i.e CGA), which are typically associated with wounding and biotic stress responses [49,50]. Therefore, it is conceivable to hypothesize that AM086 fruit cutting stimulates *de novo* biosynthesis of CGA, which is hence made available both for phytoalexins production[51]and for cell wall strengthening[52], together with the accumulation of precursors due to the suggested increased sugar catabolism, through lignin biosynthesis. Interestingly, these metabolic responses are not activated in AM199, where the absence of transcriptional induction of biosynthetic genes might be due to the already higher basal level of CGA (almost two times higher than in AM086), which might operate a metabolic regulation of PAL transcription and activity by negative feedback[53].

Distinct defensive mechanisms might be operating in AM199 line. It has been shown that, both in eggplant varieties and in tomato, wounding determines the preferential induction of group B PPOs, whose promoters are characterized by the presence of several responsive elements for wounding stress and defense response[17,29]. According to our data, group B PPOs are induced by cutting in AM086 but not in AM199, which shows only transient activation of group A PPOs, thus suggesting that different regulatory signals are activated early after cutting in this latter line. We might speculate that the post-cut rapid and intense browning of AM199 fruit flesh, paralleled by increased expression of PPO A, might be a defense response proceeding through oxidative browning of other available compounds than CGA (i.e. quinic acid), since quinones formed by oxidation of phenols exhibit direct toxicity to insects and inhibit protein digestion in herbivores by covalently binding leaf proteins.

Physical wounding is transducted in several physiological responses, by inducing respiration, cell division, and biosynthesis of plant defense compounds as well as by altering primary carbon metabolism[45]. AM199 showed a higher basal content of glycoalkaloids than AM086, nevertheless both lines reacted to mechanical injury by stimulating glycoalkaloid production, although with a different trend (Table 3). Similarly, cutting of potato tubers resulted in considerably increased glycoalkaloid content compared to the basal level[54].

In many Solanaceous species wounding or insects chewing damage determines the release of systemin, which in turn actively induces the synthesis of the proteinase inhibitors[55]. To deeper investigate the wounding signaling, we measured the expression level of the “late genes” for the proteinase inhibitors PINI and PINII (Fig 4) which, along with PPOs, act downstream the wounding signaling pathway[3,17,56]. Interestingly, the unusually fast activation of PIN II only in AM199 gives further evidence that this line reacts to wounding differently from AM086.

Taken together our results suggest that the two eggplant genetic lines are characterized by a distinct wounding perception and signaling, and that the fast and high browning of cut fruits, consequent to oxidation of phenols by PPO, the high content of glycoalkaloids, as well as the activation of proteinase inhibitors, represent the first line of defense against potential herbivore attack in AM199. On the contrary, AM086 line seems to activate a different strategy to face wounding, mostly based on the production of phenolics, both as chemical defense and as structural compounds for the enforcement of the cell wall and strengthening of mechanical barriers.

Further studies are needed to assess whether browning has an effective functional role in eggplant pathogen resistance to understand whether the low browning character might be easily transferred to other cultivars without affecting pathogen resistance.

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Figure Captions

Figure 1 Phenotypical and molecular changes associated to post cut browning A) Photograph of cut eggplant lines AM086 and AM199 showing the post-cut browning during the time course experiment.T0 corresponds to time immediately after cutting, T1 corresponds to 15 mins post cutting, and T2,corresponds to 30 mins post cutting of the eggplant fruits **B)** Gene transcript levels were detected by qRT-PCR in the fruit flesh of *S. melongena* lines AM086 and AM199 during the three time points selected to monitor the flesh browning (T0, T1 and T2).The results were analyzed using the $^{\Delta\Delta}$ Ct method and presented as fold changes compared to T0, used as internal calibrator. The *S. melongena* APRT gene was used as an internal control to normalize expression levels. The displayed values are the means ± SD of three technical replicates from three biological samples. Means separated by different letters within each eggplant line are significantly different according to Tukey test at $p< 0.05$.

Figure 2 Expression profiles of the two polyphenoloxidase gene groups identified in *S. melongena.* Transcript levels of the PPO genes group A (1, 2 and 3) and group B (4, 5, and 6) were detected by qRT-PCRin the fruit flesh of *S. melongena*linesAM086 and AM199 during the three time points selected to monitor the flesh browning (T0, T1 and T2) .The results were analyzed using the $^{\Delta\Delta}$Ct method and presented as fold changes compared to T0, used as internal calibrator. The *S. melongena* APRT gene was used as an internal control to normalize expression levels. The displayed values are the means ± SD of three technical replicates from three biological samples. Means separated by different letters within each eggplant line are significantly different according to Tukey test at $p< 0.05$.

Figure 3 Expression profiles of the Proteinase inhibitors *PinI* and *PinII* involved in the eggplant response to wounding. Gene transcript levels were detected by qRT-PCR in the fruit flesh of *S. melongena* lines AM086 and AM199 during the three time points selected to monitor the flesh browning (T0, T1 and T2).The displayed values are the means ± SD of three technical replicates from three biological samples. Means separated by different letters within each eggplant line are significantly different according to Tukey test at $p< 0.05$.

Figure 4 Chlorogenic acid and sugar content of AM086 and AM199 eggplant fruits during the time course. CGA A) and sugars B); sucrose, fructose and glucose content in the fruit flesh of *S. melongena* lines AM086 and AM199 during the three time points selected to monitor the flesh browning (T0, T1 and T2). The results were obtained through HPLC analyses. Means separated by different letters within each eggplant line are significantly different according to Tukey test at $p< 0.05$.

Table 1 Browning determination. Mean values of color L*a*b* coordinates of the flesh (center) at T0 and after 30 min (T30). For each genotype distances to pure white both at T0 (DW0) and 30 min (DW30) after fruit cutting are reported. The degree of browning (DB), calculated as difference between DW30and at DW0is reported. Color difference (CD) of the flesh calculated as Euclidean distance 30 min after the fruit cutting is also reported together with the degree of browning visually scored with a scale from 0 (minimum browning) to 4 (maximum browning).DW0 = [(100 – L0*)2 + a0*2 + b0*2)]0.5. DW30 = [(100 – L*0)2 + a*02 +b*02)]0.5. CD=[(L*30 – L*0)2 + (a*10 – a*0)2 +(b*10 – b*0)2]0.5.

Table 2 Chlorogenic acid content (CGA), polyphenoloxidase activity (PPO) and total phenols content in the ten eggplant lines depicted in figure.

Table 3 Metabolite content in AM086 and AM199 eggplant lines during the time course.

Table 4 List of primers used for qRT-PCR analyses.