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Resveratrol Biosynthesis Up-Regulates the Ascorbate/Glutathione Pathway in Transgenic Tomato Fruit

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Abstract

There is a growing interest in producing food plants with increased amounts of antioxidants because of their potential health benefits. Polyphenolic secondary metabolites, such as flavonoids and stilbenes, have been investigated for their significant antioxidant activity, important both for plant physiology and human nutrition.

With the aim to verify whether the synthesis of resveratrol in tomato fruit affected the redox status of transformed tomato tissues, in the present work we took advantage of the availability of two different transgenic tomato lines in which a grape stilbene synthase gene was expressed under a constitutive or a tissue specific promoter, respectively.

The induction of resveratrol synthesis in tomato affected the redox status of transformed tomato fruit. In particular, ascorbate and glutathione pool increased, significantly and proportionally to the amount of the resveratrol synthesised in transformed tissues. Noteworthy, the increase in ascorbate and glutathione pool paralleled with a significant increase in the activities of the main enzymes involved in redox homeostasis, i.e. catalase, ascorbate peroxidase as well the enzymes responsive for ascorbate recycling. Furthermore, lipoxygenase activity and levels of polyunsaturated fatty acids hydroperoxides, were reduced in fruit tissues from transgenic tomato lines. The synthesis of resveratrol, together with the global redox status had an impact on the total antioxidant activity of transgenic fruits.

Again the total antioxidant capability increased proportionally to the amount of synthesised resveratrol with the most remarkable increase recorded from the lipophilic-resveratrol containing-fraction of transgenic fruits. Overall, our results pointed a higher nutritional value of resveratrol synthesizing tomato fruits.

Keywords: Resveratrol; Ascorbate; Glutathione; Stilbene synthase; *Solanum lycopersicum*

Introduction

Fruit ripening is a complex genetically programmed process that results in dramatic changes in color, texture, flavor, and aroma of the fruit [1]. Due to the economic importance of fruit crop species, these processes have been, and continue to be, extensively studied at both the biochemical and genetic levels. Tomato fruit is a typical product of Mediterranean diet and has a significant part in the human diet. Hence, in addition to research plain directed to understanding and improving the organoleptic qualities of tomato fruit, significant efforts have also been devoted to increase its nutritional value.

Tomato (*Solanum lycopersicum* Mill.) contains different kind of antioxidants such as ascorbic acid (ASC), β-carotene, lycopene, lutein, and zeaxanthin [2]. In plants, ASC is highly abundant and accumulates intracellularly at concentrations ranging from 2 to 25 mM [3]. It is primarily known for its antioxidant properties, but it also acts as a cofactor for various enzymes, among which several di-oxygenases involved in peptidyl prolyl hydroxylation and phytohormones biosynthesis [4]. ASC is essential for plant growth, being involved in cell division and expansion [5-7]. It is also pivotal in the control of flowering time, seed and fruit development and senescence [3,8,9]. In addition, ASC and its oxidized form dehydroascorbic acid (DHA) act as signals in a plethora of biotic and abiotic stresses [10-12]. ASC metabolism has been reported to have nodal regulatory roles in tomato fruit development and in their protection against post-harvest stresses [13].

In animals, vitamin C serves as a cofactor for the hydroxylation of proline and pro-collagen involved in the formation of normal structures in cartilages, bones and teeth and is implicated in immune system functions, wound healing, allergic defenses, neurodegenerative and cardiovascular diseases, and even cancer [14,15]. Since, several lineages of invertebrates, insects, fish, some birds, flying mammals, and primates lack the capacity to synthesize ASC, the right levels of this metabolite within cells depend on its dietary uptake. Indeed ASC is an important vitamin, especially in populations where consumption of plant food products is limited. Therefore, the benefits arising from an increase in ASC level synthesis are relevant for the improvement of quality and nutritional values of fruits and vegetables. Furthermore, a better understanding of the mechanisms involved in the synthesis and storage of vitamin C in plants would facilitate the development of novel functional foods enriched in this vitamin. The mechanisms controlling ASC pool in tomato fruit as well as its relevance in other metabolic processes occurring during fruit maturation are still under debate. This is due to the link among ASC metabolism and other pathways involved in fruit ripening. For example, intermediates in the ASC biosynthesis are common with those of cell wall components [16]. A recent study, based on comparative transcriptomic analysis, suggested that lines containing

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QTL promoting ASC accumulation have increased L-galactonic acid availability, due to higher pectin degradation. These results underline the relevance of metabolic fluxes and precursors availability for ASC biosynthesis [17]. ASC itself promotes cell wall modification thus affecting flesh softening [18]. ASC is also pivotal for other metabolic pathways in which it is involved as substrate or cofactor, such as H_2O_2 removal by ASC peroxidases (APX) or ethylene biosynthesis, only to give a few examples [4,19]. Furthermore, apart from the reaction in which it is specifically involved, ASC interacts with other redox/ROS scavenging molecules in a network that sinergistically control redox homeostasis [20,21].

It is well known that oxidative processes take place during tomato fruit ripening: a transient increase in reactive oxygen species (ROS) production occurs during the intermediate phases of maturation [22,23]. The dynamics of ASC- and ROS- related enzymes during the process has been widely investigated [13,22].

A further element of complexity arises from our previous evidence that transgenic resveratrol producing tomato lines showed increased ASC level. These fruits, in spite of having higher levels of vitamin C, also show higher amount of glutathione (GSH) and increased anti-inflammatory capability [21,24].

Resveratrol, possess important bioactivities, including anti-inflammatory, antioxidant, anti-aggregatory functions, and modulation of lipoprotein metabolism [25]. Its chemopreventive properties against certain forms of cancer and cardiovascular disorders and positive effects on longevity and diabetes have been also reported [26-29].

However, stilbenes are found in only low quantities in the human diet, for example, resveratrol is found in low concentrations ranging from 0.3 to 7 mg aglycones/L and 15 mg glycosides/L in red wine, the most common source of resveratrol [30]. Because resveratrol is found in such small quantities in the diet, any protective effect of this molecule is unlikely at normal nutritional intakes.

Tomato fruits have been found to accumulate significant amounts of carotenoids although they are particularly poor in flavonoids [31]. This plant species does not naturally produce resveratrol, however, the precursors for resveratrol synthesis are present [21]. Therefore, the synthesis of resveratrol should be feasible in tomato plants if a stilbene synthase gene is provided [32].

Cross-talk between endogenous and novel pathways must be taken into account when producing metabolically engineered transgenic plants, as revealed by the consequences of stilbene synthase gene expression in tomato plants on stilbenes profiling and plant reproduction [25,33,34].

Since to our knowledge, no link in terms of common intermediates exists between the ASC biosynthetic pathway and resveratrol biosynthesis or catabolism, it might be hypothesised that resveratrol could substitute ASC in some unspecific reactions, partially protecting it from degradation. On the other hand, the increase in ASC recorded in resveratrol producing transgenic plants might be also the consequence of ASC biosynthesis activation as a defense mechanism aimed at protecting cell metabolism against a molecule perceived as xenobiotic.

In order to better elucidate the mechanism responsible of ASC and GSH increase in resveratrol producing tomato fruits, two transgenic lines (35SS and LoxS) were analyzed for their level of ASC, GSH, lipid peroxidation as well as for the activity of enzyme involved in ROS scavenging and ASC recycling.

To our knowledge, this is the first report concerned with the effects of resveratrol biosynthesis mediated by stilbene synthase expression on native antioxidant network in tomato fruits.

Materials and Methods

Plant material

Transgenic tomato lines were obtained from *Solanum lycopersicum* Mill. (cv. Money maker), inserting a grapevine *StSy* cDNA [35]. Two different transgenic tomato lines were considered for this work: a) the 35SS tomato line, in which the *StSy* gene was under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter; and b) the LoxS tomato line, in which the *stsy* gene was under the control of the fruit specific promoter TomLoxB, as described in Beaudoin and Rothstein (1997) [36].

Cuttings of rooted plantlets were transferred every three weeks on to a fresh medium by somatic propagation.

S. lycopersicum (cv. Moneymaker) wild type and the transgenic lines were adapted to soil and transferred in a growth chamber for 30-50 d in long-day conditions (16 h light – 28°C/8 h dark – 25°C).

To minimise sample variation, pooled samples (composed of freeze-dried powder from three tomatoes from three different plants) were considered.

Resveratrol determination by RP-HPLC-DAD

The pooled samples consisting of 0.3-0.5 g of freeze-dried powder from skin tissues were used to quantify resveratrol according to methods already described [21,24]. Independent transgenic lines were analyzed for the presence of resveratrol by reverse-phase C18 HPLC analysis. Metabolites were extracted from 0.5 to 1.0 g of fresh skin, in 100% acetone O/N. The supernatant was dried completely under nitrogen and dissolved in 0.7 ml of methanol followed by the addition of 0.3 ml of water. The sample was vortexed vigorously, sonicated for 5 min, then centrifuged to remove the insoluble debris. The final supernatant was concentrated to dryness then dissolved in 0.1 ml of methanol. Characterization of the putative resveratrol-conjugate was performed by β-glucosidase digestion of a crude skin extract. Methanol was removed under nitrogen and the residue dissolved in 200 µl of 25 mM citric acid/phosphate buffer (pH 5.2) containing 0.5 mg *per* ml of almond β-glucosidase (Sigma Chemical, St. Louis, MO). After incubation at 37°C for 1 h, the free resveratrol was extracted for three times from the aqueous phase with an equal volume of ethyl acetate. The ethyl acetate extracts were pooled and concentrated to dryness under nitrogen, dissolved in methanol, then analyzed by HPLC. The identity of resveratrol was confirmed by co-chromatography with a resveratrol standard (Sigma Chemical) and by comparison of UV absorbance spectra.

Ascorbate and glutathione determination

Analyses of ascorbate-glutathione cycle enzymes and catalase, was performed according to Paradiso et al. (2012) [9], by using a Beckman DU 7000 spectrophotometer.

0.5 g of tomato skin tissues were ground to a fine powder in a mortar in the presence of liquid nitrogen and homogenized with an extraction buffer, consisting of 50 mM Tris–HCl (pH 7.5), 0.05% cysteine and 0.1% bovine serum albumin (BSA), in a 1 : 4 ratio (w/v). The homogenates were centrifuged for 15 min at 20000 g and the supernatant was used for the enzymatic determinations.

APX activity was determined following the H_2O_2-dependent oxidation of ASC at 290 nm in a reaction mixture composed of 350 µM ASC; 170 µM H_2O_2, 50-100 µg proteins and 0.1 M phosphate buffer, pH 6.4. The activity of APX was corrected by subtracting the H_2O_2-not dependent ASC oxidation. An extinction coefficient of 2.8 mM^{-1} cm^{-1} was used.

DHA reductase activity was performed following the increase in absorbance at 265 nm due to the GSH-dependent production of ASC. The reaction mixture contained 0.1 M phosphate buffer, pH 6.2, 2 mM GSH and 50-100 µg proteins. The reaction was started upon addition of 1 mM DHA. The rate of nonenzymatic DHA reduction was subtracted (extinction coefficient 14 mM^{-1} cm^{-1}).

MDHA reductase was tested by measuring the oxidation rate of NADH at 340 nm in a reaction mixture composed of 0.2 mM NADH, 1 mM ASC, 50–100 µg proteins and 50 mM Tris-HCl, pH 8.0. The reaction was started by adding 0.2 units of ASC oxidase (Boehringer Mannheim, Germany) to generate a saturating concentration of MDHA (extinction coefficient 6 mM^{-1} cm^{-1}).

Catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase) was assayed in a reaction mixture contained 0.1 M phosphate buffer (pH 6.2), 18 mM H_2O_2 and 50-100 µg protein, with an extinction coefficient of 23.5 $mM^{-1}cm^{-1}$.

Assays of enzyme activities

Soluble proteins were extracted starting from 0.5 g skin from red tomato fruits. Homogenization conditions and protein extraction for LOX activity determination were performed as previously reported [34]. LOX activity was assayed incubating protein extracts for 10 min in 1 mL of 0.1 M sodium phosphate buffer pH 6.0 containing linoleic acid (0.3 mM final concentration). Linoleic acid hydroperoxides were quantified by RP-HPLC after reduction with sodium borohydride as above described [37]. Authentic standards of 9- and 13-hydroperoxides of linoleic and linolenic acids were purchased from Larodan (Malmö, Sweden). Calibration curves (five point's measurements) for these compounds were established.

Analyses of ASC-GSH cycle enzymes and ROS scavenging enzymes were performed as previously reported [9].

Antioxidant activity of tomato skin extracts

Total antioxidant capability was measured according to Arnao et al. (2001) [38] with minor modifications. Briefly, one gram of tomato fruit tissues was ground in a mortar with liquid nitrogen. Afterwards, 50 mM Na-phosphate buffer (pH 7.5; 2 ml) and ethyl acetate (5 ml) were added to the skin powder. The homogenate was centrifuged at 4000 g for 10 min, in order to separate the aqueous from the organic phase. The hydrophilic and lipophilic antioxidant capabilities were measured on the two phases, collected separately, by using the 2, 2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABS)/horseradish peroxidase (HRP) decolouration method. The capability of the aqueous and organic phases to scavenge the ABTS radical cautions was compared to a standard dose-response curve obtained by using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and was expressed as Trolox equivalent per fresh weight (µmol 100 g^{-1} fresh weight) [38]. The whole amount of hydrophilic and lipophilic antioxidant capabilities was considered as total antioxidant activity.

Accession number

The EMBL accession number of *Vitis vinifera* StSy cDNA is X76892.1.

Results

Resveratrol determination in transgenic tomato skin

Two transgenic tomato lines (cv Money maker), one expressing the stilbene synthase gene under the constitutive 35S (35SS) and the other under the mature fruit-specific promoter TomLoxB (LoxS), were obtained by *Agrobacterium* mediated genetic transformation of cotyledons, as previously described [21,24]. According to our previous results [24,34] the phenotype of both the transgenic lines was similar to that of the wild type, showing a regular development, flowering and fruit maturation with the remarkable difference that the 35SS tomato plants were seedless.

Our previous results revealed that the genetic modification of tomato plants led to a different capacity of synthesis and accumulation of four stilbenes (*trans*- and *cis*-piceid and *trans*- and *cis*-resveratrol) in their fruits [24]. In red fruit skin the main stilbenes produced were resveratrol glucoside and its aglycon form. Interestingly, these levels are significantly higher than those found the most common source of resveratrol in different red wines (0.3-7 mg aglycones/L and 15 mg glycosides/L in red wine) [30]. Levels of *trans*-piceid and *trans*-resveratrol in LoxS fruits were about 20-fold lower than in 35SS fruits.

Trans-resveratrol, *trans*-piceid and their correspondent *cis*-forms were quantified in the green and red fruits from both the transgenic tomato lines by RP-HPLC. These two ripening stages were chosen as characteristic phases of tomato fruit [22]. Consistently with previous analyses performed on the same transgenic plants, the levels of resveratrol varied during fruit maturation with the same trend in the skin and flesh of tomato fruits with the highest levels recorded in the skin [21,33]. For this reason the analyses here reported were carried out in the fruit skin.

RP-HPLC analysis of *trans*-resveratrol extracted from transgenic fruits skin revealed the highest level of accumulation in 35SS fruit at the red stage of ripening (about 150 µg per gram of fresh weight). In this tomato line, the hydrolysis mediated by β-glucosidase resulted in an increase in the amount of the unglycosylated metabolite at the green and red stages of about 3 (from 20 to 70 µg g^{-1} fresh weight) and 2 fold (from 100 to 160 µg g^{-1} fresh weight), respectively (Figure 1a); thus indicating that a consistent part of resveratrol is accumulated in the glycosylated form in this tissue.

The amount of *trans*-piceid and *trans*-resveratrol detected in LoxS fruits were lower than in 35SS fruits at all the analyzed stages, in particular, in green fruit of LoxS line these compounds were close to the detection limit, and increased during maturation up to the mature red stage (Figure 1b).

Effect of resveratrol synthesis on the level of ASC and GSH in green and red tomato fruits

Resveratrol biosynthesis induced an increase of ASC pool (ASC+DHA) and GSH pool (GSH+GSSG) either in the green and red stages of 35SS tomato fruits (about 60% and 100%; respectively). In the case of GSH pool the recorded increases were about 40% and 100%, in green and red stages respectively. Therefore, the increase in these metabolites paralleled the content of resveratrol in these tissues (Figure 2). In green fruits of the LoxS transgenic line, no significant differences were detected in the levels of ASC and GSH pools whereas they showed an increase in red ones in comparison with levels recorded from wild type fruits (about 25% and 35%, respectively).

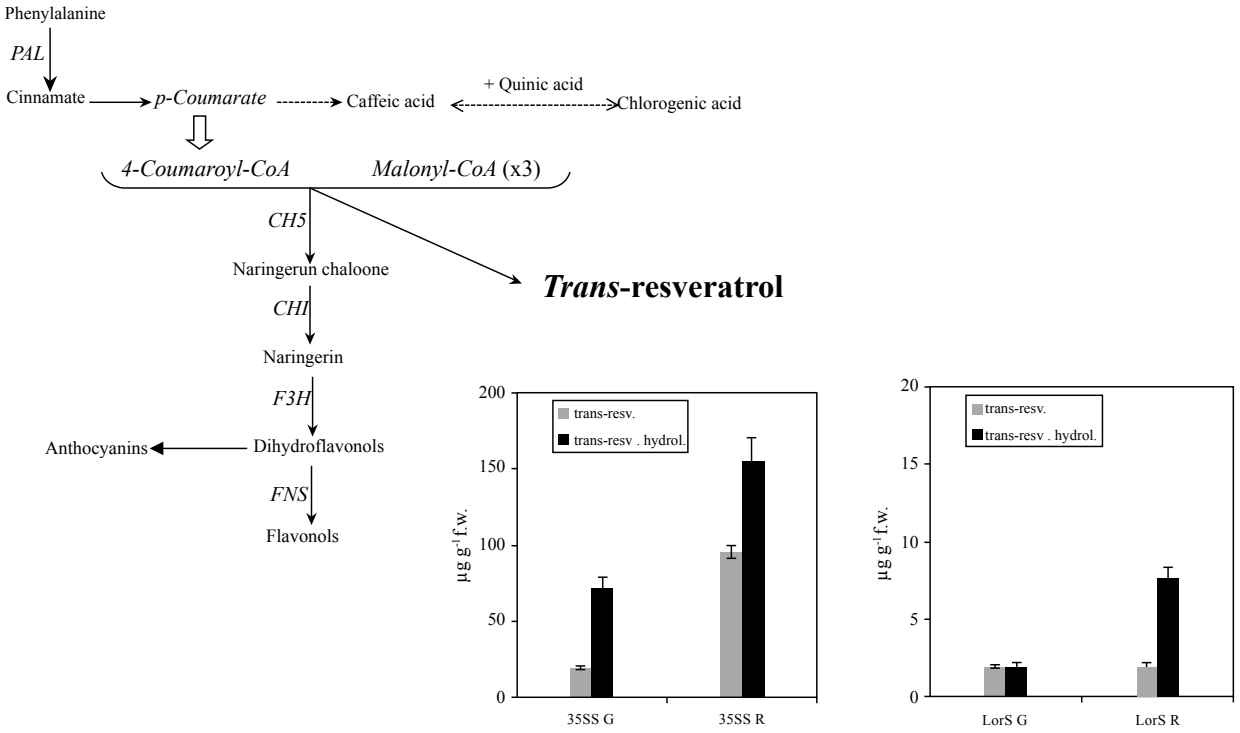

Figure 1: Resveratrol biosynthetic pathway in transgenic tomato.

The expression of a stilbene synthase gene in tomato competes with substrates involded in the first step of the complex flavonoids route. Levels of free *trans*-resveratrol before (trans-resv.) and after treatment with β−glucosidase (trans-resv. hydrol.) in fruits of 35SS (a) and LoxS (b) plants at green and red stage of ripening. The data represent means from three independent experiments.

ASC-GSH cycle enzymes and catalase

Since ROS production has been reported to occur during tomato fruit maturation [22,23], the enzymes responsible for hydrogen peroxide removal, namely catalase (CAT), ASC peroxidase (APX) as well the enzyme responsive for ASC recycling (monodehydroascorbate reductase –MDHAR - and dehydroascorbate reductase -DHAR) were analized. In wild type tomato fruits the activity of ASC related enzymes were higher in green phase than in red one, whereas CAT did not changed significantly. The activity of all these enzymes was higher in 35SS fruits, than in wild type with the highest increase recorded in red fruit (Figure 3). In LoxS fruits, an increase in the activities of these enzymes was evident only in the red phase (Figure 3).

Effects of resveratrol biosynthesis on the anti-oxidant capacity of tomato fruit

The total anti-oxidant capacity of transgenic lines and wild type fruit skins were measured using the Trolox Equivalent Anti-Oxidant Capacity (TEAC) assay.

The TEAC assay carried out on the lipophilic fraction extracted from 35SS fruit, showed a twofold increase compared to wild type fruit either at green or red stages. In LoxS fruit, a significant increase of antioxidant capability was evident in lipophilic fraction only from red fruits (Figure 4a). No significant difference were detected in the water soluble fraction (HAA) of wild type and transgenic fruits at the green stage (Figure 4b); whereas, a moderate but significant increase was evident in the 35SS and LoxS red fruit.

Lipoxygenase activity and hydroperoxydes production

Our results indicated that the lipophilic fraction of red fruit from both the transgenic lines possess a higher antioxidant capacity in comparison with wild type fruits. Furthermore, our previous results showed that levels of lipid peroxidation were reduced in 35SS red fruits [24]. Therefore, lipoxygenase (LOX) activity, a main enzyme responsible for lipid peroxidation, was monitored in mature red fruits. Results shown in figure 5a indicated that LOX activity was consistently lower in 35SS than in wild type red fruit. In LoxS red skin the LOX activity showed only a slight reduction (Figure 5a). A similar trend was recorded from skin of green tomato fruits (data not shown). The lower LOX activity detected in high resveratrol producing transgenic lines could be due to the inhibitory effect of this phyto-chemical on LOX activity, as already hypothesized in other previous reports [39].

These results were further confirmed by the analisys on the pool of hydroperoxy fatty acids (HFA) carried out on the skin of wild type and transgenic tomato fruits. Our results indicated that total HFA levels did not showed significant changes in wild type and LoxS, whereas they were significantly lower in 35SS red fruit skin (Figure 5b). Noteworthy, levels of 9-HFA increased in comparison with 13-HFA either in 35SS or in LoxS fruit skin. Indeed about 1:2 and 1:1 9/13-HFA ratio was recorded from transgenic and wild type fruit, respectively (Figure 5b). These results might indicate that different LOX isoforms were induced in transgenic tomato lines.

Discussion

Metabolic engineering has proved to be pivotal in unraveling links

between different metabolic pathways. In our previous work, we have shown that the induction of resveratrol biosynthesis in tomato plants, by genetic transformation mediated by a grape StSy gene, determines specific perturbations in the amount of the stilbenes that are end products of the phenylpropanoid pathways normally present in the fruits [25,33,34]. This is not surprising since StSy diverts part of the first intermediates of the stilbene biosynthetic pathways naturally active in the fruits, towards resveratrol biosynthesis. Furthermore, our previous results indicated that the presence of resveratrol in 35SS tomato line does not alter the accumulation trend of ASC and GSH during fruit ripening, two molecules not biosynthetically related with stilbenes, even if in 35SS whole tomato fruits the total amount of ASC and GSH is higher than in wild type [21]. Here we showed that a significant increases in ASC and GSH levels was also recorded in the skin of green and red tomato fruits constitutively expressing the StSy gene. The increase of ASC and GSH in resveratrol synthesizing tomato fruits is not due to alteration in the fluxes of metabolic intermediates, since no link have been reported among the biosynthetic pathways of these molecules. Apart their role as substrates and cofactor of specific enzymes, ASC and GSH are involved in a series of redox reaction in which they act as non-specific antioxidants [5,19]. It has been reported that, at least for ascorbate, the oxidized form is more susceptible to degradation than the reduced one [40,41]. Therefore the presence of resveratrol, a molecule with a well established antioxidant capability, might prevent the oxidation of other antioxidant molecules thus preventing their degradation. Indeed, the recorded increase of ASC and GSH was higher, in red fruits from tomato plants transformed with grape StSy under the control of 35S promoter, which also showed the highest level of resveratrol. Consistently, in LoxS transformed plants no increase in ASC and GSH occurs in green fruits where resveratrol level was very low and increased in LoxS-red fruits, were a higher amount of resveratrol, was detected (Figure 2).

The total antioxidant capability detected in the skin of transformed and wilt type fruits is coherent with the presence of resveratrol and the increased levels of ASC and GSH. In particular, the lipophilic antioxidant capability strongly increased due to the presence of resveratrol in transformed fruits, whereas the hydrophilic antioxidant capability slightly increased most likely as a consequence of the increased level of ASC and GSH (Figure 4). The small alteration induced in hydrophilic antioxidant capability of metabolically engineering tomato fruits might be explained by the presence of other hydrophilic metabolites, not affected by the presence of resveratrol, and contributing to the antioxidant capability of this fraction.

During tomato fruits maturation remarkable variations occur in the activities of the H_2O_2 scavenging enzymes APX, as well as in the ASC recycling enzymes MDHAR and DHAR. Results show in figure 3 indicated a higher activity of these enzymes in the skin of green fruits than in the red ones (Figure 3). These results are in agreement with previous ones reporting higher expression levels of APX and MDHAR encoding genes in green fruits than in red one [13]. Furthermore, a higher activity of antioxidant enzymes in immature ripening stage of tomato fruit in comparison with ripe stage has been also reported by Jimenez et al. (2002) [22]. The decrease in the activity of these enzymes during fruit ripening is coherent with a reduction of ROS producing metabolisms, since all these enzymes are involved in ROS scavenging system, directly (APX) or through the ASC-GSH cycle (MDHAR and DHAR). Indeed, it is well known that green fruit has higher production of ROS than red one [22]. This is in part due to the reduction of photosynthetic activity during tomato fruit maturation [42]. Moreover, a strong increase in mitochondrial respiration, another key ROS producing metabolism, is induced by ethylene climacteric pick in immature tomato fruits [43]. Therefore ROS scavenging pathways are pivotal for maintaining redox homeostasis in immature fruits. Our

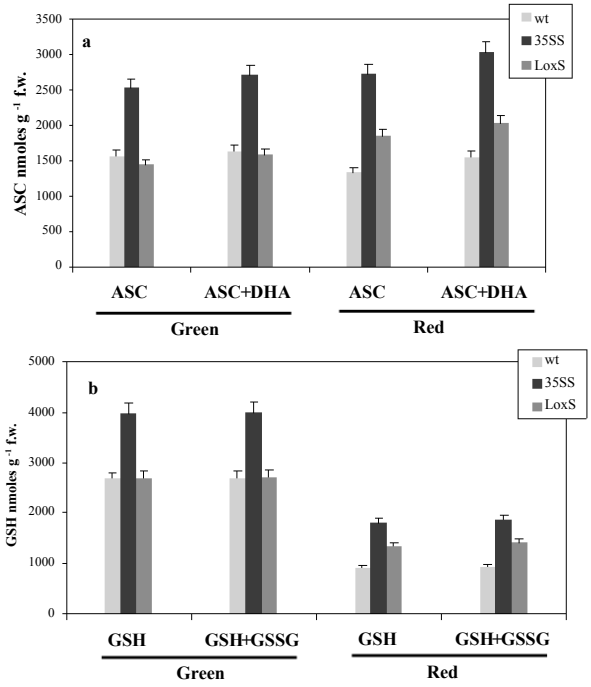

Figure 2: ASC and GSH content in green and red skin of tomato fruit.

Levels of ascorbate (**ASC**) and ascorbate pool (ASC plus dehydroascorbate: DHA) (**a**); glutathione (GSH) and glutathione pool (GSH plus glutathione disulfide: GSSG) (**b**) were analysed in tomato fruit skin from wild type and StSy transgenic lines at green (G) and red (R) ripening stages. The reported values are the means of 3 different experiments ± standard error.

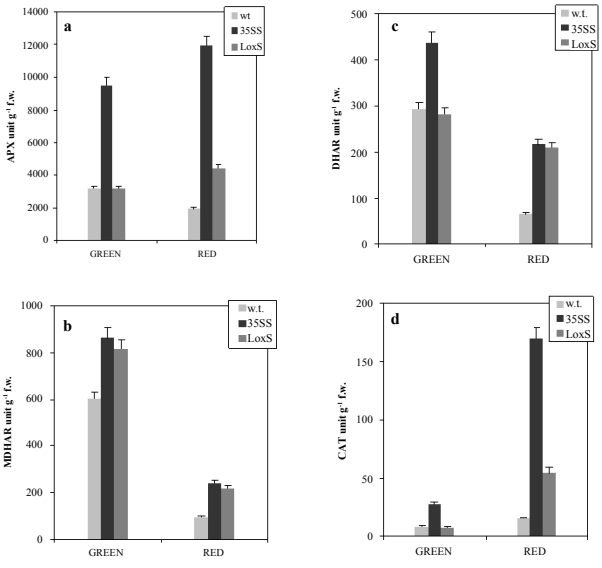

Figure 3: ASC-GSH cycle enzymes and catalase activities.

a: ASC peroxidase (APX), **b**: monodehydroascorbate reductase (MDHAR), **c**: dehydroascorbate reductase (DHAR) and **d**: catalase (CAT) activity were assayed in wild type (wt), 35SS and LoxS tomato transgenic fruit at green and red ripening stages. The reported values are the means of at least three different experiments ± standard error.

results indicated a higher CAT activity in red fruit than in green one (Figure 3). This result differs from that of Jimenez et al. (2002) [22] that reported a decrease in the activity of this enzyme during fruit ripening. The divergence of these results might be due to the different analyzed tissues: whole fruit [22] or the skin (Figure 3).

The presence of resveratrol does not alter the changes, typically occurring during maturation, in the activities of these enzymes but induces an increase proportional to resveratrol synthesis levels (Figure 3). Consistently the activity of APX, CAT, MDHAR and DHAR are higher in the skin of green and red 35SS-tomato fruits than in wild type, while in LoxS- tomato skin the increase is evident only in red phase. The increase in these ROS scavenging enzymes parallels the reduction in LOX activity and HFA levels shown in figure 5. The increase in ROS scavenger and ASC recycling enzymes could be part of the activation of a defense mechanism triggered by the presence of resveratrol that could be itself perceived as a xenobiotic molecule.

Interestingly, the analyzed enzymes play pivotal role in the defense mechanism against many kinds of stresses. An increase in these enzyme activities is often correlated with improved resistance of the plants able to better modulate their levels in response to the presence of stressing conditions [19,44-47].

In conclusion, our results suggest that metabolic engineering of phenylproanoid pathways mediated by the StSy gene in tomato results in the production of resveratrol accumulating fruits. Furthermore, increased levels of other antioxidant metabolites were also found in transgenic fruits. The higher level of ASC and GSH could confer an improved nutritional value to tomato fruits, in particular due to the increased amount of vitamin C. The presence of resveratrol together with the higher levels of ASC and GSH well explain the higher antioxidant capability and anti-inflammatory properties of 35SS tomato line [33,34].

The increase of the activities of a set of enzymes typically involved

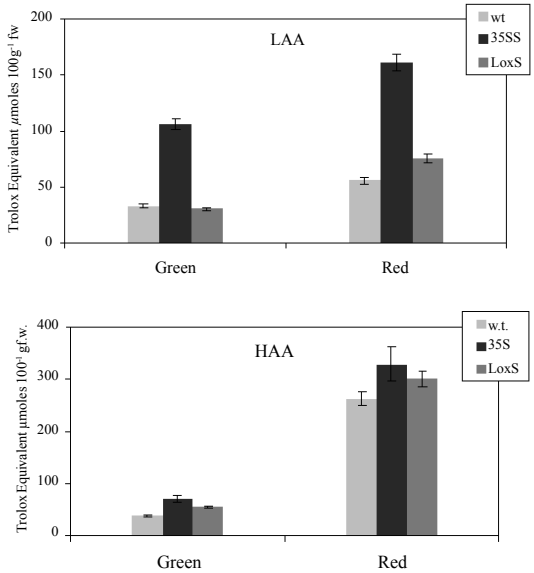

Figure 4: Antioxidant capability of green and red skin fruit.

Antioxidant capability was measured as Trolox equivalent (μmoles/100 g of fresh weight) from lipophilic (LAA) and hydrophilic (HAA) fractions of wild type (wt), 35SS and LoxS tomato transgenic fruit at green and red ripening stages. The reported values are the means of three different experiments ± standard error.

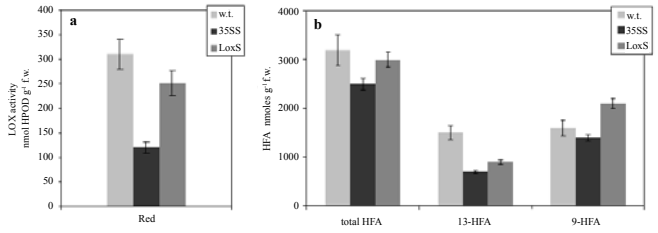

Figure 5: LOX activity and hydro(pero)xy fatty acids (HFA) in red tomato fruit.

Lipoxygenase (LOX) activity (**a**) and lipid hydro(pero)xides (HFA**)** profiling (**b**) in red fruits skin from wild type (w.t.), 35SS and LoxS tomato plant. HFA were reduced with sodium borohydride and separated by RP-HPLC and SP-HPLC. The reported values are the means of three different experiments ± standard error.

in plant defense responses in StSy metabolically engineered plants could also be of great interest in order to obtain plants with improved resistance against different kinds of stresses and also contribute to conferee improved post-harvest proprieties in tomato fruits.

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