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Antioxidant and anti-inflammatory properties of tomato fruits synthesizing different amounts of stilbenes

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Summary

Resveratrol, a plant phenolic compound, is found in grapes and red wine, but is not widely distributed in other common food sources. The pathway for resveratrol biosynthesis is well characterized. Metabolic engineering of this compound has been achieved in tomato plants (*Lycopersicon esculentum* Mill.) in order to improve their nutritional value. Tomato plants synthesizing resveratrol were obtained via the heterologous expression of a grape (*Vitis vinifera* L.) cDNA encoding for the enzyme stilbene synthase (StSy), under the control of the fruit-specific promoter TomLoxB. The resulting LoxS transgenic plants accumulated *trans*-resveratrol and *trans*-piceid, in particular in the skin of the mature fruits. Quantitative analyses carried out on LoxS fruits were compared with those of a tomato line constitutively expressing the *stsy* gene (35SS). The LoxS fruits contained levels of *trans*-resveratrol that were 20-fold lower than those previously reported for the 35SS line. The total antioxidant capability and ascorbate content in transformed fruits were also evaluated, and a significant increase in both was found in the LoxS and 35SS lines. These results could explain the higher capability of transgenic fruits to counteract the pro-inflammatory effects of phorbol ester in monocyte–macrophages via the inhibition of induced cyclo-oxygenase-2 enzyme.

Introduction

Foods of plant origin provide vitamins and a complex mixture of other substances with antioxidant activity, including polyphenol, which may be an important exogenous defence for humans against oxidative stress (Frei, 2004; Lin and Weng, 2006). It is widely accepted that the consumption of numerous chemically different antioxidant molecules is more effective for human health than the intake of only a few chemical species (Frei, 2004; Niggeweg *et al*., 2004). However, often one or a specific class of secondary metabolites is peculiar to each fruit and crop plant, in addition to those belonging to primary metabolism that are commonly present in plant tissues. However, flavonoids are widely distributed and are an integral part of the human diet, as they contribute to the determination of the colours, aromas and fragrances of numerous fresh and dried fruits, vegetables and seeds (Murcia and Martinez-Tomè, 2001; Nijveldt *et al*., 2001). Compounds biosynthetically correlated with flavonoids include resveratrol and, in general, stilbenes, but their biosynthesis is restricted to only a few plants, including grapes, where they act as phytoalexins (Bais *et al*., 2000; Jeandet *et al*., 2002). In nature, the most abundant form of resveratrol seems to be the 5,3,4′-dihydroxystilbene-3-*O*-β-D-glucopyranoside. The number and position of moieties play an important role in the biological activity of the compound (Soleas *et al*., 2001; Stivala *et al*., 2001; Halls and Yu, 2008). Experimental studies have shown that resveratrol exhibits anti-inflammatory and cardioprotective potential by inhibiting the expression of inflammatory mediators

(Subbaramaiah *et al*., 1998; Kundu *et al*., 2006) and monocyte adhesion to vascular endothelial cells (Carluccio *et al*., 2003; Csiszar *et al*., 2006). Because of their presumed health benefits, there is growing interest in the development of food crops with tailor-made levels and compositions of polyphenol, designed to exert optimal bioavailability or biological effects.

Endogenous flavonoids are present in tomato fruit tissues (Bovy *et al*., 2007). Moreover, previously, we have described tomato plants that constitutively synthesize stilbenes following the insertion of a grape structural gene encoding a stilbene synthase (StSy) enzyme. *trans*-Resveratrol and *trans*-piceid were produced in all tissues of transformed plants, with higher efficiency in fruits than in other organs (Giovinazzo *et al*., 2005). With regard to reproductive development, transformed constitutive plants produced seedless fruits. With the aim of verifying whether the modulation of the amount of resveratrol synthesis in different tomato fruit tissues has any impact on flavonoid metabolism and the antioxidant capability of fruit tissues, we report here a new transgenic line expressing the *stsy* cDNA under the control of a tissue- and developmental stage-specific lipoxygenase B gene promoter from tomato (Beaudoin and Rothstein, 1997). The availability of this new line, which accumulates 20 times less resveratrol than plants constitutively synthesizing stilbenes, allowed us to correlate the impact of different amounts of resveratrol on the redox status of the cells and the inhibitory effect on the expression of cyclo-oxygenase-2 (COX-2) in monocyte–macrophages *in vitro*.

Results

Molecular and biochemical analyses of LoxS transgenic tomato fruits

Transgenic *Lycopersicon esculentum* (cv. Moneymaker), expressing the *stsy* gene under the control of the mature fruit-specific promoter (TomLoxB), was obtained by genetic transformation through *Agrobacterium* infection of cotyledons, as described previously (Giovinazzo *et al*., 2005). We first verified the presence of the *stsy* gene in the rooted plantlets and the levels of synthesis and accumulation of the StSy protein in transgenic plants.

Figure 1 shows a schematic drawing of the cassettes harbouring a grape *stsy* cDNA under the control of TomLoxB or 35S promoters (Figure 1a,b, respectively). Polymerase chain reaction (PCR) analysis performed on DNA extracted from four independent transgenic plants showed an amplification signal of about 1350 bp corresponding to the almost complete grape cDNA (1470 bp). By contrast, no amplification was obtained when PCR was performed on genomic DNA from non-transformed plants or those transformed with an empty plasmid (Figure 1c).

The phenotype of all LoxS-transformed plants was similar to that of the wild-type, showing regular development, flowering and fruit maturation. Moreover, unlike 35SS tomato plants, which were seedless, LoxS fruits showed normal seed set, comparable with the wild-type (data not shown).

The presence of the StSy protein in transgenic plants was demonstrated by Western blot analysis on the total protein extracts from whole, mature LoxS fruits and a control wild-type plant. Using a polyclonal antiserum, raised against the recombinant StSy expressed in *Escherichia coli*, tomato lines expressing the foreign gene were identified.

As a reference for recombinant protein accumulation, an extract of transgenic fruit constitutively expressing the *stsy* gene (35SS) was also included. Western blot analysis revealed that all the extracts from LoxS fruits gave a protein band of the expected molecular mass. The highest levels of protein accumulation were detected in extracts from 1.2 plants, although these were lower than those found in 35SS fruits (Figure 1d).

The three different LoxS lines characterized above were analysed for resveratrol accumulation in mature fruits by reverse-phase high-performance liquid chromatography (RP-HPLC), as reported previously (Giovinazzo *et al*., 2005). Analysis of *trans*-resveratrol extracted from red fruits of the three LoxS lines (1.1, 1.2 and 3.3), and hydrolysed with β-glucosidase, showed comparable amounts of both *trans*-resveratrol and *trans*-piceid. The highest level of resveratrol was found in 1.2 fruits, matching the StSy protein result (Figure 1d,e). The identification of the stilbenes extracted from transgenic tomato fruits was performed on the basis of the retention time and ultraviolet (UV) spectra (Nicoletti *et al*., 2007). The results revealed that the genetic modification of tomato plants led to a different capacity for the synthesis and accumulation of four stilbenes (*trans*- and *cis*-piceid and *trans*- and *cis*-resveratrol) in fruits. The levels of *trans*-piceid and *trans*-resveratrol in LoxS fruits were about 20-fold lower than the levels in 35SS fruits (Figure 2a). The amounts of *trans*-piceid found in the skin of LoxS and 35SS fruits were 6 and 126.58 μg/g fresh weight, respectively (Figure 2c). It is interesting to note that the amount of *trans*-piceid detected in the flesh was higher in LoxS than in 35SS fruits (3.36 and 0.91 μg/g fresh weight of *trans*-piceid in LoxS and 35SS respectively; Figure 2b). The tissue specificity of the TomLoxB promoter could be responsible for StSy protein expression in the flesh; however, substrate availability in this tissue is scarce (Bovy *et al*., 2007).

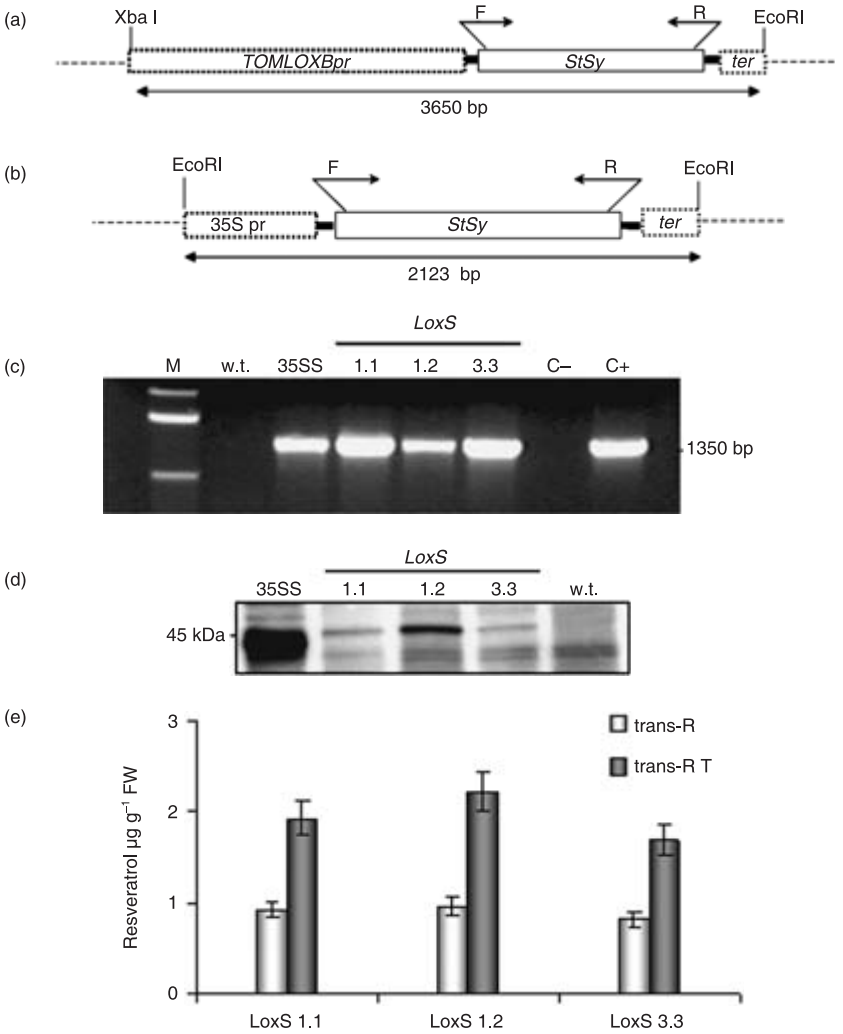

Figure 1 Schematic drawing of cassettes harbouring a grape cDNA for the stilbene synthase gene (*stsy*) under the control of TomLox and 35S promoters. (a) Plasmid containing TomLoxB-*stsy* cDNA cassette. (b) 35S-*stsy* plasmid. (c) Polymerase chain reaction (PCR) analysis of *stsy* cDNA insertion in transgenic plants. wt, wild-type plant; 35SS, constitutively transformed plant; 1.1, 1.2, 3.3, TomLox-*stsy*-expressing plants; C+, plasmid containing 35S-*stsy* cassette; C–, empty plasmid. PCR analysis performed on the DNA of four transgenic plants regenerated from independent transformation experiments shows an amplification signal of about 1350 bp, corresponding to the almost complete grape cDNA (1470 bp). The 1350-bp fragment was present in the transformed plants, but amplification was negative when PCR was performed on genomic DNA of non-transformed plants or those transformed with an empty plasmid. (d) Western blot analysis of 15 μg of total protein extracts from fruit with a stilbene synthase (StSy)-specific antibody. The hybridization signal shows a different amount of StSy protein in 35SS (constitutively transformed) plants and 1.1, 1.2 and 3.3 fruit from LoxS plants. (e) Levels of free *trans*-resveratrol before (*trans*-R) and after (*trans*-RT) digestion with β-glucosidase in red fruits of the three LoxS plants (1.1, 1.2, 3.3). No significant variation in the metabolic rate, before or after hydrolysis, was found in the independent transgenic fruit.

Ascorbate levels and total antioxidant capability

The synthesis of resveratrol and the overall changes caused by the novel branch in the flavonoid pathway also affected the ascorbate pool. Ascorbate synthesis in LoxS red whole fruit and skin was analysed and compared with that of 35SS and wild-type counterparts. Ascorbate increased in both 35SS and LoxS fruits, and paralleled the different levels of resveratrol accumulation. This increase was more evident in skin tissues (Figure 3).

We also determined the antioxidant power of both hydrosoluble and liposoluble fractions of mature fruits by Trolox equivalent antioxidant capacity (TEAC) (Arnao *et al*., 2001). As expected, no significant changes were found in the hydrosoluble fraction, whereas a remarkable increase was found in the liposoluble fraction of 35SS fruits (about three-fold higher than that of control fruits) (Figure 4a). As a consequence, the total antioxidant capability was about two-fold higher in 35SS fruits than in the wild-type (Figure 4b). In the case of LoxS fruits, there was a slight increase in the antioxidant level, which could be a consequence of the low level of resveratrol compared with that in 35SS fruits.

Anti-inflammatory capability of resveratrol-synthesizing tomato fruits

To evaluate the anti-inflammatory properties of transgenic 35SS and LoxS fruits synthesizing different levels of resveratrol, their effects on the pro-inflammatory enzyme COX-2 were analysed. With this aim, we used an *in vitro* inflammation model consisting of U937 monocytoid cells expressing the inducible COX-2 isoenzyme on stimulation with phorbol esters. To test the effect of tomato fruit extracts on COX-2 induction, U937 cells were pretreated with the extracts from 35SS and LoxS fruits for 1 h, containing about 30 and 3 μM

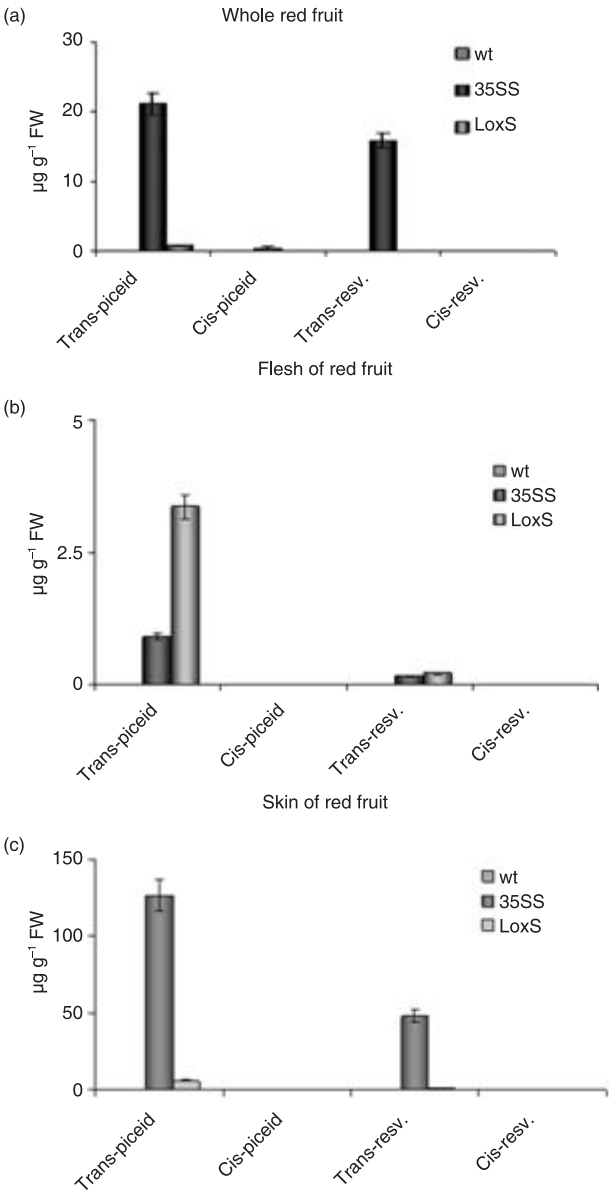

Figure 2 Stilbene accumulation in 35SS and LoxS mature whole fruits (a), flesh (b) and skin (c). The amounts of *trans*-resveratrol, *trans*-piceid and the corresponding *cis*-forms were simultaneously detected on the basis of their retention times and ultraviolet (UV) spectra of the additional peaks observed after UV irradiation at 366 nm of *trans*-resveratrol and *trans*-piceid, respectively. Results are the means of at least three independent experiments. FW, fresh weight; wt, wild-type.

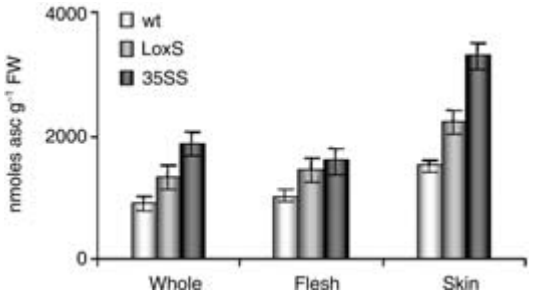

Figure 3 Comparison of ascorbate levels (nmoles per gram of fresh weight) in whole red fruit, flesh and skin of wild-type (wt) and transgenic (LoxS and 35SS) mature fruits. Results are averages from three independent experiments. FW, fresh weight; wt, wild-type.

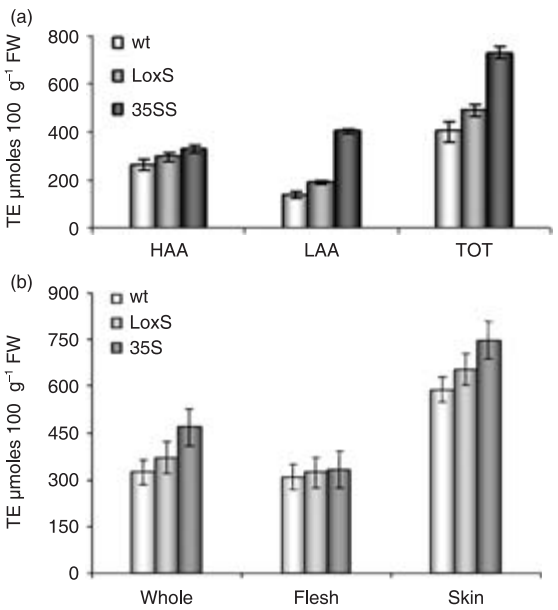

Figure 4 Characterization of antioxidant power in mature fruit from wild-type, LoxS and 35SS tomato lines. (a) Antioxidant capability of hydrosoluble fraction (HAA), liposoluble fraction (LAA) and total antioxidant capability of whole mature fruit (TOT). (b) Total antioxidant capability of whole mature fruit, flesh and skin. Results are the means of at least three independent experiments. FW, fresh weight; wt, wild-type.

of *trans*-resveratrol, respectively, before treatment with 50 nM of phorbol myristate acetate (PMA) for 16 h. As positive and negative controls, U937 cells were pretreated for 1 h with commercial pure *trans*-resveratrol or the solvent used to dissolve resveratrol before stimulation with PMA. As expected, Western analysis revealed that COX-2 protein synthesis was induced by PMA stimulation (Figure 5, lane C), whereas pure resveratrol significantly reduced the stimulated COX-2 protein (Figure 5, lane RSV). Pretreatment with the tomato extracts containing different levels of resveratrol (30 µM for 35SS and 3 µM for LoxS) reduced PMA-stimulated COX-2 expression to a greater extent than pretreatment with control extract. The decrease correlated with the level of resveratrol detected in transgenic tissue and the total antioxidant power (Figure 5). In addition, the stronger inhibitory effect displayed by the 35SS extracts compared with commercial pure resveratrol at a similar concentration suggests an additive role caused by the presence of other phytochemicals detected in transformed tomato extracts

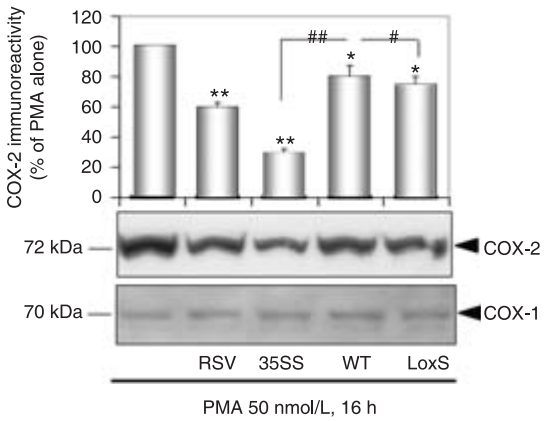

Figure 5 Resveratrol-synthesizing tomato inhibited phorbol myristate acetate (PMA)-stimulated cyclo-oxygenase-2 (COX-2) expression in monocytoid cells. U937 cells were pretreated with vehicle (ethanol), resveratrol-synthesizing tomato fruits (35SS, LoxS) or wild-type fruit for 1 h and then stimulated with PMA for 16 h. Cell extracts, with equal amounts of proteins, were immunoblotted with monoclonal antibodies against COX-1 or COX-2. Top panel shows the densitometric analysis of COX-2 immunoreactivity expressed as the percentage of PMA alone. These results were obtained from three independent experiments with similar results. $*P < 0.05$ and $**P < 0.01$ compared with PMA-treated cells; $\#P < 0.05$ and $\#\#P < 0.01$ compared with wild-type fruit-treated cells. WT, wild-type.

(Figure 5). The effect of tomato extracts or pure resveratrol appeared to be specific for COX-2, as they did not modify the level of the housekeeping enzyme COX-1 (Figure 5). No cytotoxic effect was induced by these treatments (data not shown).

Discussion and conclusions

The beneficial effect of *trans*-resveratrol on human health has directed research towards the production of this metabolite in staple plants (Fremont, 2000; Schijlen *et al*., 2006). This study was undertaken to determine the potential influence of the modulation of fruit phenols, and the consequent pleiotropic effects, on metabolites, and other effects of transgenesis.

Recently, we have reported that the constitutive expression of the *stsy* gene induces the synthesis of stilbenes in whole fruit and even in the flesh, where the production of polyphenol is usually scarce (Giovinazzo *et al*., 2005; Nicoletti *et al*., 2007). The phenotype of transformed plants was similar to that of the wild-type, showing regular development, flowering and fruit ripening, although the fruits were seedless. These results suggest that the synthesis of resveratrol in flower tissues is able to induce a change in the flavonoid pathway. The influence of this pathway on fertility has been reported in tobacco and tomato (Fisher *et al*., 1997; Schijlen *et al*., 2007).

In this study, we have shown that plant transformation by an *stsy* cDNA under the control of a fruit tissue- and development-specific promoter (TomLoxB) triggers the expression of StSy and the synthesis of resveratrol only in the edible part of tomato plants (data not shown).

The most efficient transcriptional activity of this promoter was found in the skin and outer flesh of red fruits (Kausch and Handa, 1997). Consistent with this finding, LoxS plants expressed the *stsy* gene in mature fruit tissues, and accumulated resveratrol in a detectable amount preferentially at the red stage of ripening (data not shown). However, LoxS fruits produced 20-fold less resveratrol than constitutively transformed fruits. This allowed us to compare the effect of different amounts of resveratrol on fruit phenotype, antioxidant capability and, finally, the anti-inflammatory process.

Resveratrol has a well-known antioxidant capability and may also affect the metabolism of molecules that are functionally correlated (Murcia and Martinez-Tomè, 2001), especially in some physiological stages of fruit ripening when oxidative processes occur (Brady, 1987; Jimenez *et al*., 2002). Our data suggest that the acquisition of a novel branch of flavonoid metabolism in tomato fruits, which finally leads to resveratrol, induces a significant increase in ascorbate (Figure 3), with higher levels recorded from the skin of 35SS than LoxS fruits. As the result reflects the variation in the level of resveratrol synthesis in the different transformed lines and tissues, it is possible that resveratrol replaces ascorbate in some antioxidative processes that occur during fruit ripening, which do not require specific electron donor molecules.

Metabolic changes induced by resveratrol synthesis in fruits determine an increase in the total antioxidant capability (Figure 5). This feature can be mostly attributed to the liposoluble fraction, which is probably closely related to the synthesis of *trans*-resveratrol. Indeed, *trans*-resveratrol seems to have a higher antioxidant activity in comparison with many other compounds, such as ascorbate and α-tocopherol (Rice-Evans *et al*., 1997). It has been reported previously that tocopherol levels are not affected in resveratrol-synthesizing tomato fruits (Giovinazzo *et al*., 2005), although it is well known that ascorbate regenerates tocopherols from their oxidized form (Smirnoff, 2000). Therefore, the increase in ascorbate content in transformed fruits could also indirectly affect their lipophilic antioxidant properties. The increase in liposoluble antioxidant power in transformed fruits is also consistent with the evidence that the levels of lipid peroxidation are significantly lower in resveratrol-synthesizing tomato fruits than in wild-type fruits (Giovinazzo *et al*., 2005).

The slight increase in the hydrophilic fraction detected in transgenic fruits was not unexpected, as the antioxidant power of glycosylated polyphenols is lower than that of aglycon forms (Murcia and Martinez-Tomè, 2001). With regard to hydrosoluble intermediates of the phenylpropanoid pathway, further work is in progress to analyse different classes of intermediates, although our preliminary results indicate that the overall changes induced by resveratrol determine some perturbations at specific levels along the pathway (D'Introno *et al*., unpubl. data).

Changes induced by resveratrol in the flavonoid pathway can be explained by considering that resveratrol can act either as an antioxidant or a phytoalexin. As the products of the pathway produce antioxidant molecules, it is important to understand how one molecule may affect the other, and the extent of cross-talk between the two.

Several studies have reported that resveratrol has cardioprotective effects because of its ability to increase plasmatic antioxidant capacity, inhibit platelet aggregation and coagulation, reduce low-density lipoprotein oxidation and suppress the pro-inflammatory response (Pace-Asciack *et al*., 1996). In addition, resveratrol displays chemopreventive potential by inhibiting the growth of several types of tumour in animal models (Jang *et al*., 1997). The anti-tumorigenic, anti-inflammatory and cardioprotective effects of resveratrol seem to be related, at least in part, to its ability to suppress prostaglandin production through its interference with the expression and activity of COX-2, the rate-limiting enzyme in prostaglandin biosynthesis. COX catalyses the synthesis of prostaglandins from arachidonic acid. There are two COX isoforms, designated COX-1 and COX-2. COX-1 is expressed constitutively in most tissues and appears to be responsible for housekeeping functions (Vane *et al*., 1998). In contrast, COX-2 is not detectable in most normal tissues, but is induced by oncogenes, growth factors, carcinogens and tumour promoters (DuBois *et al*., 1994; Subbaramaiah *et al*., 1998). In particular, macrophages are prominent producers of prostaglandin during inflammatory processes in response to signals that trigger macrophage activation, such as bacterial lipopolysaccharide or phorbol ester (Beloqui *et al*., 2005). Altered COX-2 levels and consequent abnormally high prostaglandin secretion are thought to be involved in diverse pathological processes, and COX-2-specific inhibitors represent important challenges for cancer treatment, as well as chronic inflammatory diseases such as atherosclerosis (Warner and Mitchell, 2004). The effects of resveratrol-enriched tomato extracts on COX-2 expression induced by phorbol ester in monocyte–macrophage U937 cells indicate that resveratrol reduces the level of the inducible, but not constitutive, COX isoform, thus confirming and expanding the anti-inflammatory activity of resveratrol (Figure 5), as suggested previously (Subbaramaiah *et al*., 1998; Carluccio *et al*., 2003, 2007; Csiszar *et al*., 2006). Remarkably, the extracts of transgenic tomato fruits containing resveratrol display an anti-inflammatory effect greater than that of chemically synthesized resveratrol or extract from wild-type fruits.

In conclusion, our results indicate that the presence of a new biosynthetic route responsible for resveratrol biosynthesis improves the health-giving biological activities of tomatoes. Both 35SS and LoxS fruits, which contained different levels of resveratrol, showed higher antioxidant and anti-inflammatory properties than wild-type fruits.

Experimental procedures

Plant material

Lycopersicon esculentum L. (cv. Moneymaker) fruit tissues were utilized in all experiments. The grape *StSy* cDNA (Sparvoli *et al*., 1994), under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter and TomLoxB promoter (Beaudoin and Rothstein, 1997), was inserted in the binary vector (pBI101) and utilized for transformation experiments. Details of the method used to produce transgenic tomatoes and their propagation have been described by Giovinazzo *et al*. (2005). All LoxS plants were self-pollinated to produce fruits.

Harvest of transgenic material

Samples of both transgenic and wild-type tomato fruits were obtained from three pairs of plants grown in a glasshouse. Three mature fruits were harvested from each plant. The outer layer (2–3 mm thick) was separated from the fruit and classified as skin. The remainder of the fruit, without seeds and jelly-like parenchyma, was classified as flesh. Separated fruit tissues and whole fruit were frozen in liquid nitrogen and stored at –80 °C until use. To minimize sample variation, pooled samples consisted of freeze-dried powder from three individual fruits, each from a different plant.

Molecular analysis of transgenic tomato

Lines of transgenic *L. esculentum* (cv. Moneymaker), expressing a grape StSy cDNA (Sparvoli *et al*., 1994) under the control of the mature fruit-specific promoter TomLoxB (Beaudoin and Rothstein, 1997), were obtained by genetic transformation through *Agrobacterium* infection of cotyledons, as described previously (Giovinazzo *et al*., 2005).

The presence of the *stsy* gene in the transgenic tomato plant genome was detected by PCR analysis using the forward primer sts1 (5′-GAGATGGTATAATTTTCTTC-3′) and reverse primer sts2 (5′-GGGATCCGATTAACTATTTAAACAGAAAT-3′). Amplification reactions contained 200 μg of genomic DNA, 1 × buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1 μM of each primer and 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Monza, Italy). The reaction mix was incubated at 95 °C for 5 min,

and then subjected to 35 amplification cycles (95 °C for 45 s, 52 °C for 45 s and 72 °C for 2 min), and completed with a final 10-min extension at 72 °C. PCR products were analysed on a 1% agarose gel in 1 × TRIS-ACETATE-EDTA BUFFER (see Figure 1c).

Total soluble proteins were extracted from tomato tissues, separated (15 μg) by 15% (w/v) sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then blotted on to a nylon membrane (Hybond-C extra, Amersham Biosciences, Milan, Italy), as described previously (Giovinazzo *et al*., 2005). Hybridization was performed by incubating the membrane with a rabbit polyclonal antiserum raised against recombinant StSy protein (Giorcelli *et al*., 2004). To detect hybridization signals, the 'Intensity Chemiluminescence System' (ECL™, Amersham Biosciences) was used.

Resveratrol extraction and determination

The pooled sample consisted of 0.5 g of freeze-dried powder from three individual fruits, each from a different plant. Resveratrol was extracted as described by Giovinazzo *et al*. (2005). All compounds were identified by their characteristic UV spectra and compared with those of authentic standards. Resveratrol was purchased from ICN Biomedicals (Irvine, CA, USA). Identification of the *trans*-resveratrol glucoside form was performed as described by Nicoletti *et al*. (2007).

Ascorbate determination

Tomato fruit (0.5 g) frozen in liquid nitrogen and ground to a fine powder was mixed with 3 mL of metaphosphoric acid 5% (w/v) in order to obtain a deproteinized extract, and manually homogenized in a mortar. The homogenate was then centrifuged at 20 000 ***g*** for 15 min and the supernatant was used for the assay. Ascorbate was determined according to dePinto *et al*. (1999).

Antioxidant activity of tomato fruit extracts

The TEAC assay was carried out according to Arnao *et al*. (2001), with minor modifications, to measure the antioxidant power in mature red fruit. One gram of tomato fruit was ground in a mortar in liquid nitrogen, and then extracted with 2 mL of 50 mM sodium phosphate buffer (pH 7.5) and 5 mL of ethyl acetate. 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 in the two phases, collected separately, according to Arnao *et al*. (2001). The capability of the aqueous and organic phases to scavenge the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) radical cations was compared with a standard dose–response curve obtained using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and was expressed as Trolox equivalents of fresh weight (μmol/100 g fresh weight). The combined hydrophilic and lipophilic antioxidant capabilities were considered as the total antioxidant activity.

Human cell culture, stimulation protocol and protein analysis

Human monocytoid U937 cells were obtained from the American Tissue Culture Collection, and grown in RPMI medium 1640 (Gibco, Milan, Italy) containing 10% (v/v) fetal calf serum (FCS), 2 mmol/L glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. To test the effect of resveratrol or resveratrol-enriched tomato extracts, 1×10^6 cells/mL per well in 24-well plates (Corning Inc., NY, USA) were shifted to 5% FCS medium at least 3 h before the addition of the designated compounds or solvent. After 60 min of treatment, U937 cells were stimulated with 50 nmol/L PMA for 16 h and then processed. After stimulation, cells were washed twice with phosphate-buffered saline (PBS) and lysed in 150 μL of lysis buffer [150 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), pH 7.9, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Triton-X100, 10% glycerol, 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulphonylfluoride (PMSF), 1 mg/mL aprotinin, 1 mg/mL leupeptin. Lysates were vortexed three times for 10 s, and incubated on ice for 30 min, after which they were centrifuged at 10 000 ***g*** for 20 min at 4 °C. After determining the protein concentration, the supernatants were stored at –20 °C until analysis. Equal amounts of proteins were separated by SDS-PAGE. The resolved proteins were transferred on to supported nitrocellulose sheets (Amersham Biosciences) and, after saturation of nonspecific binding sites, were incubated overnight with specific monoclonal antibodies against COX-1 and COX-2, both from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein bands were visualized by an enhanced chemiluminescence (ECL) Kit (Amersham Biosciences). Blots were scanned, and digitized images were submitted to densitometry analysis using Scion Image software.

Cell viability was determined by a 3-(4,5-dimethyl-thiazol)-2,5-diphenyltetrazolium bromide (MTT) method.

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