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Characterization of a strawberry late-expressed and fruit-specific peptide methionine sulphoxide reductase

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We have cloned and characterized a cDNA clone, called *Fapmsr*, coding for a putative peptide methionine sulphoxide [Met(O)] reductase (PMSR, EC 1.8.4.6) from strawberry fruits (*Fragaria x ananassa*). This gene is involved in the repair of inactive peptides and proteins caused for the oxidation of methionine residues to Met(O). Expression of the *Fapmsr* was only detected in the receptacles of red mature fruits and not in young or immature fruits nor in other plant tissues such as flowers, leaves, runners, roots or achenes. Expression of the *Fapmsr* gene was activated in green immature fruits when achenes were removed from receptacles, and this was prevented by the application of exogenous auxins such as naphthaleneacetic acid. The enzyme produced and purified by cloning the strawberry cDNA in frame with the C-terminal sequence of the glutathione S-transferase gene can reduce free Met(O) to methionine as analysed by reverse phase high performance liquid chromatography. We have also set up a PMSR protection assay that demonstrates that this enzyme can protect in vivo against the damage produced by the addition of H_2O_2.

Introduction

Protein oxidation is a frequent occurrence in cells and results in fragmentation or carbamylation of the peptide backbone (Vogt 1995). The sulphur-containing amino acids are especially susceptible to oxidation; the oxidation of methionine residues in protein to methionine sulphoxide [Met(O)] is a well-understood post-translational modification. Some of the biological oxidants of methionine residues to Met(O) in proteins are hydrogen peroxide, hydroperoxyl derivates of fatty acids and oxidized linoleic acid (Mohensin and Lee 1989). The conversion of methionine residues to Met(O) can inactivate proteins and peptides, making them lose their biological activity (Brot and Weissbach 1991). There are examples of proteins susceptible of this kind of damage such as lipoxygenase, calmodulin, phosphoglucomutase and α1-proteinase inhibitor (Brot and Weissbach 1991). The Met(O) residue can be reduced back to methionine, and this change restores the activity of these proteins by the enzyme peptide Met(O) reductase (PMSR). The enzymatic repair of oxidized proteins has been recognized as a key process in a wide range of organisms including bacteria, plants and humans (Moskovitz et al. 1996, 1997). As a consequence, it has been suggested that PMSR should be part of the minimal gene set of organisms (El Hassouni et al. 1999). An enzyme, initially described in *Escherichia coli* (called MsrA), can reduce Met(O) to Met (Brot et al. 1981). After that, PMSR activity has been found in a variety of organisms that include plants, yeast and humans (Brot and

Abbreviations – Met(O), methionine sulphoxide; NAA, naphthaleneacetic acid; PMSR, peptide methionine sulphoxide reductase; QRT-PCR, quantitative real time-PCR.

Weissbach 1991, Sánchez et al. 1983). PMSR activity was also found in germinating seeds and leaves of castor bean, spinach and in the tubers of potato (Sánchez et al. 1983). Most of the activity appears in the chloroplast and a minor activity in the cytosol.

In plants, there are few studies about the physiological role played by PMSR.

Some studies have indicated that PMSR proteins must play an important role in protecting cells against oxidative damage during the infection by pathogens and under salt stress (Sadanandom et al. 2000, Zhao et al. 2003).

Recently, a comprehensive investigation of gene expression using cDNA microarrays was carried out along the strawberry fruit ripening (Aharoni et al. 2002). In this study, the importance of the oxidative stress in the ripening process was examined. At least 20 ripening-induced genes were also induced by oxidative stress (Aharoni et al. 2002). The authors propose that, in this process, the expression of some genes is induced to cope with oxidative stress conditions produced during ripening (Aharoni et al. 2002). Alternatively, it was also proposed that the strawberry-ripening transcriptional program is an oxidative stress-induced process (Aharoni et al. 2002). The results also showed that not all the processes associated with strawberry ripening are under the same genetic control and that they are probably a collection of processes regulated in a discrete manner (Aharoni et al. 2002). Similarly, in grape (*Vitis vinifera*), the accumulation of 10 mRNAs corresponding to putative stress response proteins was recently reported (Davies and Robinson 2000).

A close relationship between the need of protecting against oxidative stress producing protein damage and *pmsr* gene induction was proposed (Bechtold et al. 2004, Gustavsson et al. 2002, Sadanandom et al. 2000).

Our research was done to characterize PMSR and determine its role during development and growth of strawberry fruit. In this article, we have cloned, characterized and sequenced a gene coding for a putative strawberry *Fapmsr* cDNA. A fusion protein was overexpressed and purified using *E. coli*. The purified PMSR protein led us to determine the putative in vivo and in vitro role of PMSR activity.

Materials and methods

Plant material

The strawberry fruits (*Fragaria x ananassa* Duch. Cv. Chandler, an octaploid cultivar) were grown in controlled fields under natural conditions. Extreme caution was taken to avoid collecting fruits with signals of fungi or bacterial infestation, in which the pattern of expression of any gene of interest could be altered. Fruits were collected and immediately frozen in liquid nitrogen and kept at −80°C until use. We classified the strawberry according to the various fruit growth and development stages as small-sized green fruits (G1), middle-sized green fruits (G2), full-sized green fruits (G3), white fruits (W), turning-stage fruits (T) and full-ripe red fruits (R).

RNA isolation and double-stranded cDNA generation

Total RNA from a pool of 6–7 fruits at different fruit growth and development stages and from roots (Rt), leaves (L), flowers (F) and runners (Ru) was isolated according to Manning (1991). Poly (A^+) RNA was obtained using an Oligo-dT cellulose column (Speirs and Longhurdt 1993).

Double-stranded cDNA was obtained from poly (A^+) RNA using a cDNA synthesis System Plus Kit (Amersham, UK) and according to manufacturer's instructions.

Cloning of the PMSR cDNA

The *Fapmsr* cDNA was isolated after differential screening of a cDNA subtractive library (full-ripe red stage vs. green-2 stage) generated by a MAST-polymerase chain reaction (PCR) method (Medina-Escobar et al. 1997a) which was cloned in the λzapII vector. Phages from plates showing differential signals were further chosen after running a secondary and tertiary PCR-based Southern blot differential screening as described by Medina-Escobar et al. (1997a). Purified positives phages were excised according to manufacturer's conditions, and the corresponding pBluescript derivative clones were isolated. Films were exposed 2 or 3 days with X-Omat films and two intensifier screens. Alternatively, a Molecular Image device (Bio-Rad, Hercules, CA) was used, which also allowed us to quantitatively assess the intensity of hybridizations.

DNA extraction

Strawberry genomic DNA was extracted as follows: Young leaves were kept in distilled water in the dark at 4°C for 2 days. After that, 2 g of leaves were ground under liquid nitrogen into a fine powder and re-suspended in 25 ml of warm (65°C) buffer solution [50 m*M* Tris-HCl pH 8, 100 m*M* NaCl, 50 m*M* ethylenediaminetetraacetic acid (EDTA), 1% (v/v) 2-mercaptoethanol, 4% (w/v) sodium dodecyl sulphate (SDS), 6% (w/v) polyvinylpolypyrrolidone (PVPP)]. The 2-mercaptoethanol, SDS and PVPP were added just before use. The mixture was incubated at 65°C with gentle rotation

for 1 h. Then, 8 ml of 3 *M* potassium acetate pH 4.8 was added, and the mixture was incubated on ice for 30 min and then centrifuged at 7000 *g* for 10 min. The supernatant mixture was filtered through a double layer of miracloth. Two volumes of ice-cold ethanol were added, and the DNA was recovered, washed 2–3 times in fresh ice-cold ethanol and dried at room temperature. The DNA was re-suspended in water.

Auxin treatment

Achenes of two sets of G2 strawberry fruits on the growing plant were carefully removed using the tip of a scalpel blade. One set of de-achened fruits were treated with the synthetic auxin naphthaleneacetic acid (NAA) as a lanolin paste with 1 m*M* NAA in dimethysulphoxide 1% (v/v). The other set of de-achened fruits (control group) was treated with the same paste but without NAA. Both the treatments were applied over the whole fruit surface. Fruits were harvested at 0, 24, 48, 72 and 96 h after treatment, immediately frozen in liquid nitrogen and stored at −80°C.

Oxidative stress treatments

For the oxidative stress experiments, white stage fruits (attached to the plants) were carefully injected using a hypodermic syringe with a needle of gauge, with 1 ml of a sterile solution of 0.3 m*M* plumbagine, 1 m*M* menadione, 0.5 m*M* hydrogen peroxide and 0.5 m*M* copper sulphate. Control fruits were injected with 1 ml of sterile water. Afterwards, fruits were harvested after 2, 4, 8 and 24 h, immediately frozen in liquid nitrogen and stored at −80°C until RNA isolation.

Northern analysis

The cDNA insert from the *Fapmsr* clone was used as template to synthesize a *Fapmsr* radioactive probe in order to investigate the differential expression of this gene during the strawberry-ripening process by Northern analysis. A cDNA corresponding to 18S ribosomal RNA was also used as a radioactive ribosomal probe to control equal loading of RNA samples. The probes were labelled to a specific activity of *ca.* 10^8 cpm μg^{-1} using an oligolabelling kit (Pharmacia, Uppsala, Sweden). PMSR antisense RNA was synthesized using T3 RNA polymerase (Boehringer, Ingelheim, Germany) and [^{32}p] UTP.

Twenty-five micrograms of total RNA per sample were used for the Northern experiments. Filters (Hybond N^+, Amersham) were pre-hybridized at 65°C for 2 h in the hybridizing solution (0.25 *M* NaH_2PO_4, 0.1 m*M* EDTA, 0.7% SDS). The probes were added to the same solution, and hybridization was carried out at 65°C for 16–18 h. Filters were washed twice in ×0.1 SSC, 0.1% (w/v) SDS at 65°C for 15 min and then exposed to an X-ray film at −80°C for 48 h.

Quantitative real time-PCR analysis

The Quantitative real time-PCR (QRT-PCR) studies of gene expression were carried out as previously described for other strawberry fruit-ripening-related genes (Benítez-Burraco et al. 2003, Blanco-Portales et al. 2002).

In QRT-PCR analysis, quantification is based on Ct (threshold cycle) values. Each reaction was done by triplicate, and the corresponding Ct values were determined. The Ct values of each QRT-PCR reaction were normalized using the Ct value corresponding to a strawberry *18S-26S interspacer* gene. Moreover, the efficiency of each QRT-PCR was also calculated. All these values were utilized to determine the increases in gene expression according to the following expression:

Fold change $= 2^{-\Delta(\Delta Ct)}$

$\Delta C_t = C_t$ ('target' gene) $- C_t$ ('housekeeping' gene)

and $\Delta(\Delta C_t) = \Delta C_t$ (condition 1) $- \Delta C_t$ (condition 2)

Southern blot analysis

Genomic DNA (5 μg) was digested with the appropriate restriction enzymes, fractionated on 0.7% (v/v) agarose gels and transferred to Hybond N^+ membranes according to protocols supplied by Amersham. DNA was fixed by UV light using a Stratalinker device (Stratagen, La Jolla, CA), and the blot was hybridized using *Fapmsr* cDNA as a radioactive probe. Hybridization and washed steps were performed as the Northern experiments.

DNA sequence manipulations

Sequencing of the cDNA clone was performed in an Applied Biosystem 310 Automatic Sequencer. Unidirectional deletions were carried out with the ExoIII/S1 method (Henikoff 1984). DNA computer analysis was performed with the GCG package (Devereux et al. 1984). Further comparisons with the current databases were also performed with the advanced Blast2 service provided by the EMBL outstation (http://www.embl-heidelberg.de:8080/blast2).

Construction of a glutathione-S-transferase-PMSR fusion protein

A fusion protein was constructed by cloning the strawberry *Fapmsr* cDNA in frame with the carboxyl-terminal

sequence corresponding to the glutathione-S-transferase (GST) present in the plasmid pGEX-KG (Smith and Johnson 1988). To do this fusion protein, we previously digested the plasmid pGEX-KG with *Xba*I, and after that, it was treated with Klenow fragment in the presence of 0.5 m*M* of dNTP, and blunt ends were made to it. On the other hand, the *Fapmsr* cDNA was digested with *BamH*I restriction enzyme and again treated with klenow as described above to make blunt ends. This cDNA was ligated to the plasmid restoring the *BamH*I site of the cDNA. In the resulting plasmid (pGEX-PMSR), the *Fapmsr* gene was fused to the GST gene under control of isopropyl-β-D-thiogalactopyranoside (IPTG) inducible *lac* promoter. Both restriction analysis and nucleotide sequence determination confirmed that the *Fapmsr* gene is ligated to pGEX-KG at the correct site and in frame with the GST gene.

XL1-blue *E. coli* cells were transformed with this construct, grown in LB medium up to the mid exponential phase of growth (A_{600nm} = 0.8) and induced for the GST-PMSR fusion protein by adding 1 m*M* of IPTG for an additional 4 h. When needed, extracts were obtained by passing cells through a French press set at 1200 psi in buffer A (20 m*M* Tris-HCl pH 7.4, 10 m*M* $MgCl_2$) containing PMSF (0.2 m*M*), EDTA-Na_2 (2 m*M*) and β-mercaptoethanol [0.1% (v/v)] and were centrifuged at 12 000 *g* for 15 min. This fusion protein was purified using a glutathione-agarose gel as previously described (Smith and Johnson 1988). The expression of this fusion protein was analysed by SDS-PAGE electrophoresis in 10% (v/v) polyacrylamide gels (Laemmli 1970) and stained with Coomassie Blue.

The PMSR was purified essentially in one step by glutathione-agarose affinity binding as described by Smith and Johnson (1988).

Disk inhibition assay

A disk inhibition assay was devised as follow: XL1-blue *E. coli* cells harbouring the plasmid pGEX-PMSR coding the GST-PMSR fusion protein were grown to log phase in LB medium (Sambrook et al. 1989) and then diluted in LB medium; these cells were grown in the present of either 1 *mM* of IPTG (inducing conditions) or 30 *mM* glucose (repressive conditions) for an additional 4 h, and then, they were poured into plates of LB medium containing the same concentrations of IPTG or glucose. A filter disk impregnated with either 10 µl of 250 *mM* H_2O_2 or 10 µl of H_2O was placed in the middle of the plate. The plates were incubated overnight at 37°C. As control, the same experiment was performed using either untransforming XL1-blue *E. coli* cells or those containing the pGEX-KG plasmid which was only expressing the GST protein.

Enzyme activity

The capability of PMSR to reduce free Met(O) to methionine was analysed (Brot and Weissbach 1991, Sánchez et al. 1983). Purified PMSR was always used in these assays. Reaction was carried out by adding 5 µg of PMSR-purified protein in 100 µl of final volume containing 20 m*M* Tris-HCl, 10 m*M* $MgCl_2$, 30 m*M* KCl buffer pH 7.5. As electron donor, 15 m*M* dithiothreitol (DTT) was used. The reaction was initiated with the addition of 0.7 m*M* Met(O) as substrate and was maintained during 1 h at 30 °C. All the reactions were stopped by adding 200 µl of 99% (v/v) ethanol and by the incubation during 10 min on ice. After centrifugation at 10 000 *g* in eppendorf tubes, 100 µl of supernatants were dansylated and analysed by reverse phase high performance liquid chromatography (HPLC) using Spherisorb ODS2 column (Teknochroma, Madrid, Spain, 15 × 0.46 cm, 5 µm) following previously described method (Márquez et al. 1986).

Results

Sequence comparison and structural features of the putative PMSR protein from strawberry

After a differential screening of a strawberry subtractive library of cDNA, more than 100 ripening-related clones were identified (Medina-Escobar et al. 1997a). We selected one of these clones for further characterization. This cDNA clone contained a 0.9-kb insert which was sequenced to completion. This cDNA (GenBank accession number z69596) showed an open reading frame region coding for a protein of 191 amino acids, with a predicted Mr of 21 600 and a pI of 7.02. Sequence comparisons with the current databases showed that this cDNA has high homology with known putative PMSR found in other plants, mammals and prokaryotes (data not shown). The identities at the amino acid levels are very high in PMSR-like sequences from plants as Arabidopsis (73%) and tomato (83%). These significant identities suggest that the isolated cDNA correspond to an mRNA encoding a PMSR protein. This clone has a 3′ untranslated region of 297 bp including 16 residues of the poly (A^+) tail, which contained a putative AATAA polyadenilation consensus site. No sequences corresponding to a signal peptide was found in the N-terminal end of the sequence nor specific carboxyl terminal sequences were found indicating that these sequences could be located in any of the organella. These data, along the highly hydrophilic profile of the protein (data not shown), are consistent with the strawberry PMSR protein being soluble and globular and located at the cytoplasm.

Southern blot analysis

We have detected several DNA fragments that hybridized on Southern blot of genomic strawberry DNA that was digested with the appropriate restriction enzymes. The pattern of hybridization bands suggests that the strawberry putative *Fapmsr* is probably encoded by at least two genes (Fig. 1). This can be explained if we take into account that *F. x ananassa* is a hybrid plant coming from the cross between *Fragaria virginiana* and *Fragaria chiloensis* and could also be related with the ploidy level of *F. x ananassa* (octaploid).

Gene expression studies

Fapmsr is a fruit-ripening-related and auxin-regulated gene

The spatial and temporal expression pattern of the *Fapmsr* gene has been studied both by Northern blot and QRT-PCR. Using the Northern blot approach, the *Fapmsr* mRNA was only detected in full red ripen fruits (Fig. 2A). No expression was detected in unripe green or white turning fruits. No expression levels in roots, flowers, runners and leaves were observed either (data not shown). A QRT-PCR (Fig. 2B,C) analysis revealed that the amount of *Fapmsr* transcript exhibited a substantial increase of the expression during the fruit ripening. The highest expression was detected when the fruit receptacle was totally mature (R stage). These results are in accordance with other ripening-related genes in strawberry fruits (Benítez-Burraco et al. 2003, Blanco-

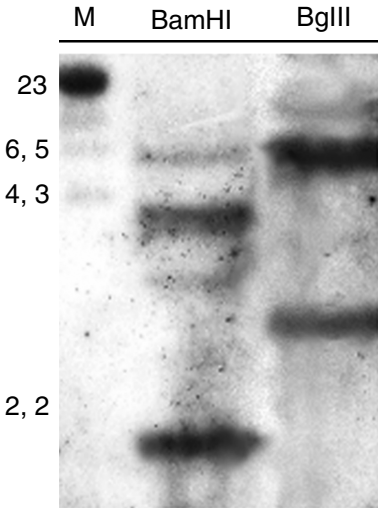

Fig. 1. Southern blot analysis of *Fragaria x ananassa* cv. Chandler genomic DNA (2 μg) digested with several restriction enzymes and hybridized with ^{32}P-labelled *Fapmsr* cDNA probe.

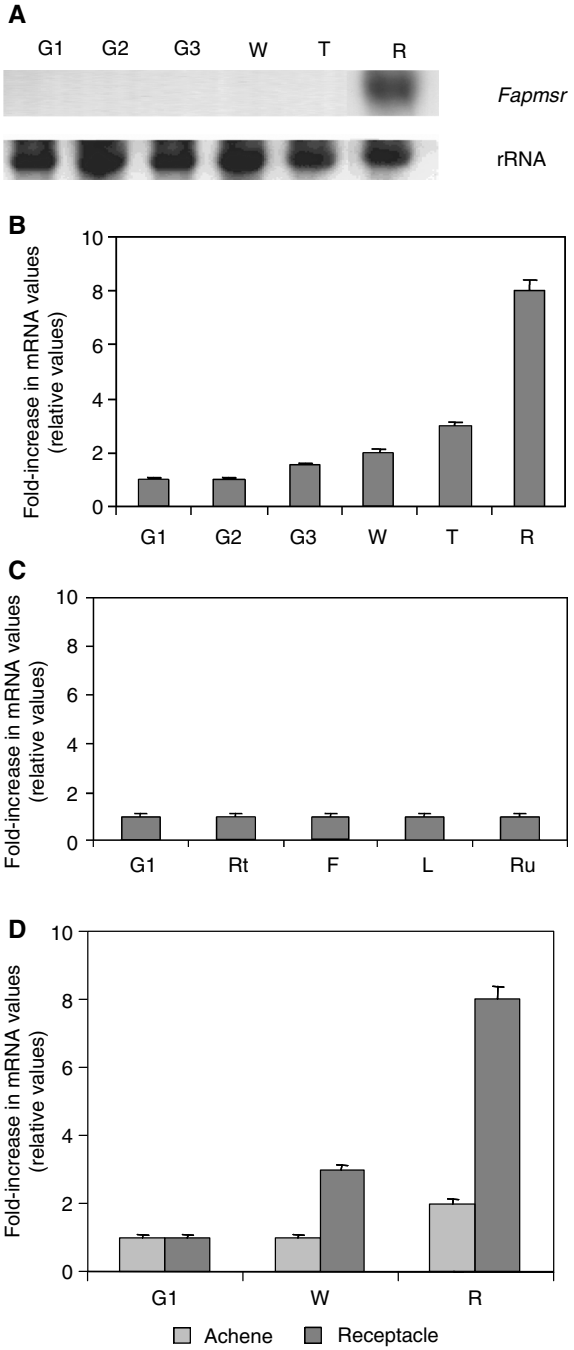

Fig. 2. Developmental and spatial expression of the *Fapmsr* gene. (A) Northern analysis of 25 μg total RNA isolated from pooled samples of G1, G2, G3, W, T and R strawberry fruit-ripening stages, using *Fapmsr* cDNA clone as probe. rRNA hybridization was used as charge control. (B) Expression studies by quantitative real time-polymerase chain reaction (QRT-PCR) from strawberry fruit-ripening stages. (C) Expression studies by QRT-PCR from vegetative tissues using G1-stage as reference. (D) Expression studies by QRT-PCR from receptacles and achenes corresponding to G1, W and R stages. Expression quantification is based on Ct values as described in *Materials and methods*. Each QRT-PCR reaction was normalized using the Ct values corresponding to a strawberry *18S-26S interspacer* gene.

Portales et al. 2002). This expression took place along all stages of fruit growth and development, both in the achene and in the receptacle tissues, although a lesser expression was observed in achenes (Fig. 2D).

Some strawberry fruit-specific genes have been shown to be under the control of auxins. To test whether the expression of the *Fapmsr* gene was also under the control of this hormone, we have analysed the gene expression on treated de-achened green fruit (G2 stage) and without NAA treatment. A clear increase in gene expression was detected in fruits after 4 days of removal of the achenes (Fig. 3). However, these increments in the *Fapmsr* transcript levels were partially prevented in G2 de-achened fruits that were treated with NAA. These results indicated that the expression of the *Fapmsr* gene is regulated by auxins as had been previously demonstrated in other strawberry fruit-ripening-related genes (Aharoni et al. 2002, Benítez-Burraco et al. 2003, Blanco-Portales et al. 2002, Manning 1998, Medina-Escobar et al. 1997b, Moyano et al. 1998, Trainotti et al. 1999).

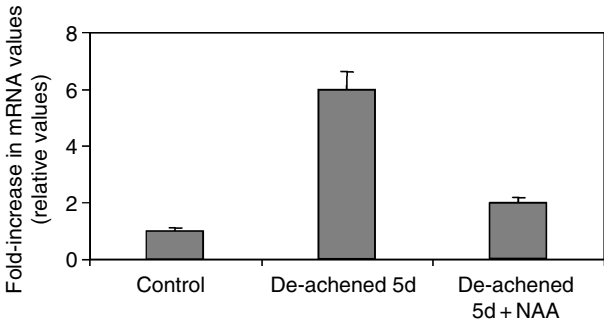

Fig. 3. Effect of removing achenes and auxin treatment on putative peptide methionine sulphoxide reductase gene expression. Expression studies by quantitative real time-polymerase chain reaction from G2-de-achened strawberry fruit after removing the achenes at 5d and G2-de-achened strawberry fruit after 5d of naphthaleneacetic acid (NAA) treatment. Control indicates G2-stage fruits with achenes. Expression quantification was carried out as described in Fig. 2.

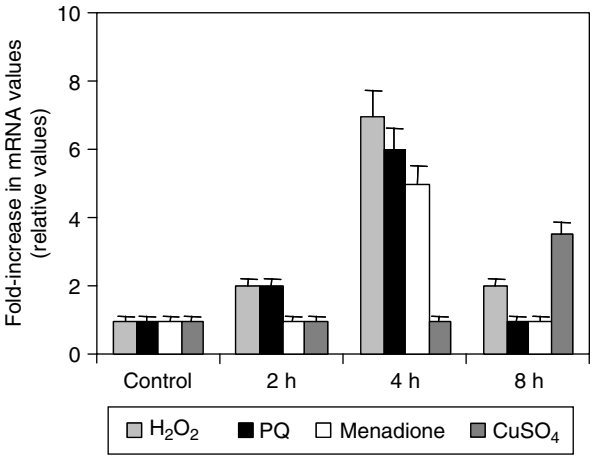

Fig. 4. The effect of oxidative stress on *Fapmsr* gene expression. Expression studies of *Fapmsr* gene by quantitative real time-polymerase chain reaction (QRT-PCR) from W-stage fruits injected with H_2O_2, paraquat (PQ), menadione and $CuSO_4$ at 2, 4 and 8 h. Control indicates W-stage fruits injected with sterilized distiller water. The quantification of the expression was carried out as described in Fig. 2.

The expression of the *Fapmsr* gene is induced by oxidative stress

Preliminary studies have demonstrated a relationship between oxidative stress and PMSR gene induction. To determine if *Fapmsr* gene expression is under the influence of the oxidative stress conditions, we have carried out experiments with free radical-generating compounds such as H_2O_2, paraquat, menadione, plumbagine and copper ions. The expression of the *Fapmsr* gene was clearly induced by all these oxidative stress treatments (Fig. 4). Similar results were obtained by other strawberry-ripening-related genes (Aharoni et al. 2002), supporting the possibility that the oxidative stress can play an important role in the strawberry fruit-ripening process (Aharoni et al. 2002).

Purification of PMSR protein after over expression of GST-PMSR fusion protein

A SDS-PAGE (Fig. 5A) analysis of XL1-blue *E. coli* cells transformed with the plasmid pGEX-PMSR that contain the fusion protein GST-PMSR shows that a soluble 46 kDa fusion protein was highly expressed only when the cells were induced with IPTG, and expression was not detected when the cells were incubated in the presence of glucose.

The PMSR was purified (Fig. 5B) essentially in one step by glutathione-agarose affinity binding as described by Smith and Johnson (1988).

PMSR activity

To determine if the strawberry PMSR was able to use free L-Met(O) as substrate, we used purified strawberry PMSR protein taken from *E. coli* cells. The amino acids were labelled with dansyl chloride and monitored by HPLC. The results are shown in Fig. 6. Fig. 6A shows the retention time of DTT and DTT contaminants. The commercial Met has a retention time of 35 min and Met(O) at 68 min (Fig. 6B). When Met(O) was added as substrate in the presence of purified strawberry PMSR protein and DTT (Fig. 6C), the peak corresponding to the Met(O) disappeared, and new peak appeared with a

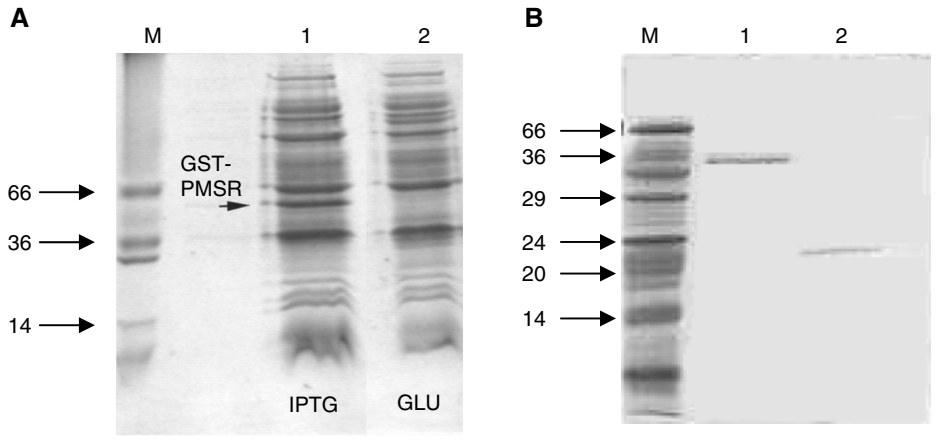

Fig. 5. (A) SDS-PAGE analysis of XL1-blue *Escherichia coli* cells transform with the plasmid pGEX-peptide methionine sulphoxide reductase (PMSR) that contain the fusion protein glutathione-S-transferase (GST)-PMSR. Lane M is the molecular marker. Lane 1 shows that the fusion protein is only expressed when the cells are induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (1 m*M*) and lane 2 shows that no induction of the fusion protein occurs when the same cells are treated with glucose (Glu). (B) SDS-PAGE analysis of purification of recombinant PMSR protein in *E. coli*. Lane M is the molecular marker, the size of protein is in KDa. Lane 1 shows the fusion protein GST-PMSR after affinity chromatography with glutathione agarose. Lane 2 shows purified PMSR after removal GST by thrombin.

retention time of 35 min corresponding to free Met. These results indicated that our purified PMSR protein was able to reduce free L-Met(O) to Met and needed DTT to do it, because in DTT absence, no Met was formed. The DTT was incapable of reducing Met(O) by itself (data not shown). This is in agreement with Sánchez et al. (1983), who demonstrated that a direct reduction of L-Met(O) cannot take place by the only presence of DTT.

Effect on growth and sensitivity to oxidative stress of *E. coli* cells containing the fusion protein GST-PMSR

The ability of this protein to reduce Met(O) residues in proteins suggested that it might be involved in protecting cells against oxidative damage (Moskovitz et al. 1995). To test this possibility, we devised an in vivo disk inhibition assay.

Fig. 7 shows the growth of *E. coli* bacterial cells containing the chimeric fusion protein when they were incubated in solid LB medium in the presence of a disk paper containing 10 µl of 250 m*M* of H_2O_2 that was placed in the surface of the medium. In the presence of glucose, when no expression of the GST-PMSR fusion protein takes place, a clear halo of inhibition of growth was observed. This halo of inhibition was not observed when the bacteria were incubated in the presence of IPTG. No differences were observed when the paper disk contained H_2O, and this was used as a control. To determine if the absence of inhibition growth was actually due to the presence of a fusion protein having PMSR activity, bacterial cells containing only the GST gene and lacking the PMSR coding sequence were grown under the same conditions. The inhibition of the growth took place in the presence of H_2O_2, in the presence of both IPTG and glucose, indicating that PMSR activity was truly required to protect cells against the oxidative stress because of H_2O_2. All these results clearly indicate that the GST-PMSR fusion protein retain PMSR activity.

Discussion

In this article, we report the isolation for the first time in non-climateric fruits, of a strawberry clone highly homologous to putative PMSR genes found in many different organisms including plants, mammals and bacteria. The importance of PMSR activity has been deeply characterized in both mammal and bacterial systems. Little is known, however, about the role of this activity in plants. The oxidation of Met to Met(O) results in the loss of biological activity of many proteins (Brot and Weissbach 1991). It has also been shown that this biological activity could be restored when the residues of Met(O) are reduced back to Met (Abrams et al. 1981). The inactivation of α1-proteinase inhibitor because of the oxidation of two critical Met residues to Met(O) has been implicated in the destruction of lung tissue in smokers' emphysemas (Carp et al. 1982). An extremely high level of Met(O) residues in cataractous lens proteins that could be associative with the etiology of the disease has also been described (Garner et al. 1983), because many photochemical reactions occur in these

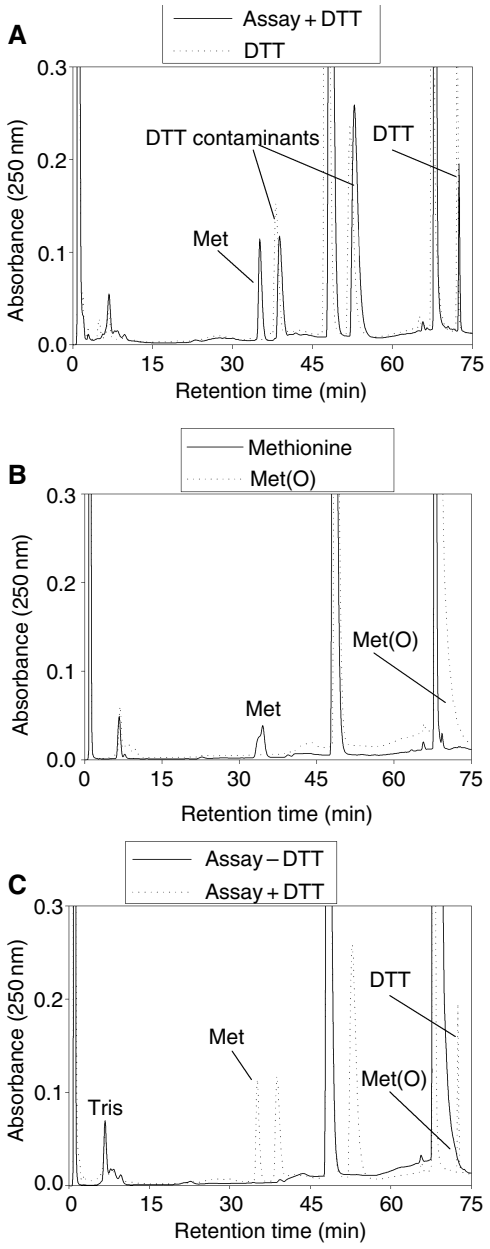

Fig. 6. High performance liquid chromatography (HPLC) analysis of peptide methionine sulphoxide reductase activity. (A) Chromatogram obtained after reverse phase HPLC corresponding to the different components of the test [Tris and dithiothreitol (DTT)]. (B) Chromatogram obtained after reverse phase HPLC corresponding to commercial Met and Met(O). (C) Chromatogram obtained after reverse phase HPLC where the discontinuous lane is the assay in the presence of DTT as donor electron shows the disappearance of Met(O) and appearance of a peak corresponding to Met. The continuous lane is the same assay without DTT and shows the maintenance of the peak corresponding to Met(O).

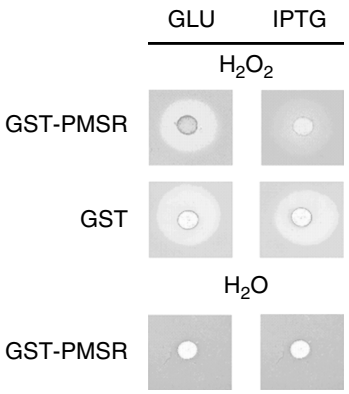

Fig. 7. Protection assay in vivo of the fusion protein glutathione-S-transferase-peptide methionine sulphoxide reductase (GST-PMSR) in *Escherichia coli*. The first column indicates LB plates with 0.5% of glucose (Glu). The second column shows LB plates with 1 m*M* of isopropyl-β-D-thiogalactopyranoside (IPTG). GST-PMSR indicates XL1-blue *E. coli* cells transformed with the plasmid pGEX-PMSR that contains the fusion protein GST-PMSR. These cells were grown overnight and spread onto LB plates supplemented with glucose or IPTG in the presence of H_2O_2 and H_2O added in the middle of a paper disk. GST indicates XL1-blue *E. coli* cells transformed with the plasmid pGEX that only contains GST protein. The figure shows the inhibition of growth due to toxicity of H_2O_2. Inhibition of the growth in the presence of H_2O was not observed.

tissues. In fact, flavins and other photo sensible compounds present in these lens tissue cause the in vivo oxidation of sulphur proteins in the presence of light (Varma et al. 1979). The normal lens probably has mechanisms to avoid photochemical damage to its structural proteins. Both vitamin C and superoxide dismutase activity can protect these lenses against the oxidative damage, but in the cataractous lens, such systems were lacking or inactive. Met(O) residues are also involved in adult respiratory distress syndrome and rheumatoid arthritis (Cochrane et al. 1983).

The main difference between plants and other organisms is that plants are the only organisms containing multiple PMSR isoforms. Four PMSR genes, three encoding cytosolic isoforms (PMSR1 to PMSR3) and one encoding a plastidial isoform (PMSR4, formerly called pPMSR) have been identified in *Arabidopsis* (Sadanandom and Murphy 1997).

Sánchez et al. (1983) and Fergusson and Burke (1994) have measured the amount and the distribution of PMSR as well as the enzymatic reduction of Met(O) to Met in plants. They showed that most PMSR activity was confined within the chloroplast. Supported by the fact that full ripe strawberry fruits lack active chloroplasts (Seymour et al. 1993), this enzyme probably is not located within the chloroplast. In addition, no *Fapmsr* hybridization signals were found in photosynthetic tissues such as leaves. Moreover, it is noteworthy to indicate that the expression of the strawberry *Fapmsr* is confined to the fruit organ and was only detected in red

ripe strawberry fruits. This expression differs from the *Arabidopsis pmsr3* (Sadanandom et al. 2000) in which all tissues expressed the mRNA encoding the *pmsr3* with the highest expression taking place in roots and leaves.

It has been proposed that the auxins may be the primary hormones controlling the strawberry fruit-ripening process (Perkins-Veazie 1995). In particular, a gradual decline in the supply of auxin from achenes in the latter stages of fruit growth (transition between green and white fruits) has been associated with the strawberry fruit ripening (Perkins-Veazie 1995). In this sense, a substantial increase in gene expression of strawberry-ripening-related genes by a lack of auxins has been described (Aharoni et al. 2002, Benítez-Burraco et al. 2003, Blanco-Portales et al. 2002, Trainotti et al. 1999). Accordingly, the strawberry *Fapmsr* gene expression is negatively regulated by auxins. Thus, the high gene expression levels found in strawberry-ripe stages and the fact that *Fapmsr* gene expression is negatively regulated by auxins clearly show that *Fapmsr* gene is another gene whose pattern of expression correlates with a normal fruit-ripening expression pattern.

At present, no endogenous substrate proteins to the pPMSR have been identified. However, in a recent report, the reduction of several highly conserved Met(O) residues by a pPMSR in the *Arabidopsis thaliana* chloroplast-localized small heat shock protein Hsp21 has been described (Gustavsson et al. 2002). This reduction led to recovery of Hsp21 conformation and of its chaperone-like activity, both of which are lost upon methionine sulphoxidation (Gustavsson et al. 2002). We have also shown here that our strawberry PMSR is capable to reduce free L-Met(O) to Met, suggesting that FaPMSR could work in recovering damaged protein through the reduction of the Met(O) groups. It is not easy to explain why PMSR activity is induced in these fruits and only at the final stage of ripening. In plants, PMSR is a ubiquitous enzyme that repairs oxidized damaged proteins. In *A. thaliana*, a cytosolic PMSR2 protein has been related with protection against oxidative stress (Bechtold et al. 2004). The proposed physiological role of this PMSR2 is the repairing of damaged proteins at the end of the night in a short day diurnal cycle (Bechtold et al. 2004). A null mutation in PMSR2 (pmsr2-1) produced high levels of oxidative stress (Bechtold et al. 2004). Although PMSR2 is not absolutely required for the viability of plants, the observation of increased damage to proteins in these long nights suggests that the timing of expression of PMSR2 is an important adaptation for conservation of their resources. Like other PMSR systems, we have demonstrated that the strawberry PMSR is capable to protect *E. coli* cells against oxidative stress, when we induced PMSR activity found in the fusion protein GST-PMSR by adding IPTG.

In this sense, recently, a cDNA microarray study has shown that the ripening of the strawberry fruit and oxidative stress conditions in the fruit induce the expression of genes encoding oxidative-stress detoxifying enzymes and proteins such as GST, glutaredoxin quinone reductase (a putative *TED2* orthologous) and ferritin (Aharoni et al. 2002). A potential source of oxidative stress could be the lignifying of the vascular tissue (Aharoni et al. 2002). In this study, it was proposed that the development of the vascular system is a significant event coupled to fruit maturation (Aharoni et al. 2002). The biosynthesis of lignin involves the formation of phenoxy radicals through the action of a wide range of oxidative enzymes, such as peroxidases, lacasses and/or by a redox shuttle-mediated enzymatic oxidation (Boudet 1998, Önnerud et al. 2002). This process occurs through a dehydrogenative polymerization via radical-coupling reactions in which hydrogen peroxide is involved. In this sense, the presence of the PMSR enzyme in lignifying vascular tissue could be related with a protective role of this enzyme to cope with an excess of oxidative stress at the last stages of fruit ripening. The clear increase of the *Fapmsr* gene expression in fruits treated with compounds generating oxidative stress conditions indicates that the enzyme coded by the *Fapmsr* gene could be involved in a protein protective role to keep these proteins functionally active under conditions of oxidative stress.

On the other hand, it has been described that the oxidized linoleic acid is capable of oxidizing Met residues and inactivate proteins in vitro (Mohensin and Lee 1989). The linoleic and other fatty acids are present in mature strawberries (Yamashita et al. 1976, 1977). These compounds are at least partially responsible for the organoleptic properties of this fruit. This opens the possibility that in strawberry fruits, these compounds could also be inactivating proteins by oxidizing methionine residues. In this case, PMSR activity would be necessary to reactivate important protein activities, but mostly when these flavour compounds are formed.

References

Abrams WR, Weissbach L, Weissbach H, Weinbaum G, Brot N (1981) Enzymatic reduction of oxidised alpha-1-proteinase inhibitor restores biological activity. Proc Natl Academic Sci USA 78: 7483–7486

Aharoni A, Keizer LCP, Van Den Broeck HC, Blanco-Portales R, Muñoz-Blanco J, Bois G, Smit P, De Vos RCH, O'Conell AP (2002) Novel insight into vascular, stress, and auxin-dependent and -independent gene expression programs in strawberry, a non-climateric fruit. Plant Physiol 129: 1019–1031

Bechtold U, Murphy DJ, Mullineaux PM (2004) Arabidopsis peptide methionine sulfoxide reductase2 prevents cellular oxidative damage in long nights. Plant Cell 16: 908–919

Benítez-Burraco A, Blanco-Portales R, Redondo-Nevado J, Bellido ML, Moyano E, Caballero JL, Muñoz-Blanco J (2003) Cloning and characterization of two ripening-related strawberry (*Fragaria x ananassa* cv. Chandler) pectate lyase genes. J Exp Bot 54: 633–645

Blanco-Portales R, Medina-Escobar N, López-Ráez JA, González-Reyes JA, Villalba JM, Moyano E, Caballero JL, Muñoz-Blanco J (2002) Cloning, expression and immunolocalization pattern of a cinnamyl alcohol dehydrogenase gene from strawberry (*Fragaria x ananassa* cv. Chandler). J Exp Bot 53: 1723–1734

Boudet AM (1998) A new view of lignification. Trends Plant Sci 3: 67–71

Brot N, Weissbach H (1991) The biochemistry of methionine sulfoxide residues in proteins. Biofactors 3: 91–96

Brot N, Weissbach L, Werth J, Weissbach H (1981) Enzymatic reduction of protein-bound methionine sulfoxide. Proc Natl Acad Sci USA 78: 2155–2158

Brot N, Werth J, Koster D, Weissbach H (1982) Reduction of N-acetyl methionine sulfoxide: a simple assay for peptide methionine sulfoxide reductase. Anal Biochem 122: 291–294

Carp H, Miller J, Hoidal R, Janoff A (1982) Potential mechanism of emphysema: α1 proteinase inhibitor recovered from lungs of cigarette smokers contains oxidized methionine and has decreased elastase inhibitory capacity. Proc Natl Acad Sci USA 79: 2041–2045

Cochrane CG, Spragg R, Revak SB (1983) Pathogenesis of the adult respiratory distress syndrome. Evidence of the oxidant activity in bronqueoalveolar lavage fluid. J Clin Invest 71: 754–761

Davies C, Robinson SP (2000) Differential screening indicates a dramatic change in mRNA profiles during grape berry ripening. Cloning and characterization of cDNAs encoding putative cell wall and stress response proteins. Plant Physiol 122: 803–812

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the Vax. Nucleic Acid Res 12: 387–395

El Hassouni ME, Chambost JP, Expert D, van Gijsegem F, Barras F (1999) The minimal gene set member msrA, encoding peptide methionine sulfoxide reductase, is a virulence determinant of plant pathogen *Erwinia chrysanthemi*. Proc Natl Acad Sci USA 96: 887–892

Fergusson DL, Burke JJ (1994) Methionyl sulphoxide content and protein-methionine-S-oxide reductase in response to water deficits or high temperature. Physiol Plant 90: 253–258

Garner WH, Garner MH, Spector A (1983) H_2O_2-induced uncoupling of bovine lens Na^+, K^+ ATPase. Proc Natl Acad Sci USA 77: 1274–1279

Gustavsson N, Kokke BP, Harndahl U, Silow M, Bechtold U, Poghosyan Z, Murphy D, Boelens WC, Sundby C (2002) A peptide methionine sulfoxide reductase highly expressed in photosynthetic tissue in *Arabidopsis thaliana* can protect the chaperone-like activity of a chloroplast-localized small heat shock protein. Plant J 29: 545–553

Henikoff S (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28: 351–359

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 251: 614–616

Manning K (1991) Isolation of nucleic acids from plants by differential solvent precipitation. Anal Biochem 195: 45–50

Manning K (1998) Isolation of a set of ripening-related genes from strawberry: their identification and possible relationship to fruit quality traits. Planta 205: 622–631

Márquez FJ, Quesada A, Sánchez-Jimenez F, Nunez de Castro I (1986) Determination of 27 dansyl amino acid derivatives in biological fluids by reversed phase high performance liquid chromatography. J Chromatogr 380: 275–283

Medina-Escobar N, Cárdenas J, Moyano E, Caballero JL, Muñoz-Blanco J (1997b) Cloning, molecular characterization and expression pattern of a strawberry ripening-specific cDNA with sequence homology to pectate lyase from higher plants. Plant Mol Biol 34: 867–877

Medina-Escobar N, Cárdenas J, Valpuesta V, Muñoz-Blanco J, Caballero JL (1997a) Cloning and characterization of cDNAs from genes differentially expressed during the strawberry fruit ripening process by a MAST-PCR-SBDS method. Anal Biochem 248: 288–296

Mohensin V, Lee JB (1989) Oxidation of α1 proteinase inhibitor: role of lipid peroxidation products. J Appl Physiol 66: 2211–2215

Moskovitz J, Rahman MA, Strassman J, Yancey SO, Kushner SR, Brot N, Weissbach H (1995) *Escherichia coli* peptide methionine sulfoxide reductase gene: Regulation of expression and role in protecting against oxidative damage. J Bacteriol 177: 502–507

Moskovitz J, Weissbach H, Brot N (1996) Cloning and expression of a mammalian gene involve in reduction of methionine sulfoxide residues in protein. Proc Natl Acad Sci USA 73: 2095–2099

Moskovitz J, Bertlett B, Poston JM, Stadtman ER (1997) The yeast peptide methionine sulfoxide reductase functions as an antioxidant in vivo. Proc Natl Acad Sci USA 94: 9585–9589

Moyano E, Portero-Robles I, Medina-Escobar N, Valpuesta V, Muñoz-Blanco J, Caballero JL (1998) A fruit-specific putative dihydroflavonol 4-reductase gene is differentially expressed in strawberry during the ripening process. Plant Physiol 117: 711–716

Önnerud H, Zhang L, Gellerstedt G, Henriksson G (2002) Polymerization of monolignols by redox shuttle-mediated

enzymatic oxidation: a new model in lignin biosynthesis I. Plant Cell 14: 1953–1962
Perkins-Veazie P (1995) Growth and ripening of strawberry fruit. Hort Rev 17: 267–297
Sadanandom A, Murphy DJ (1997) A novel form of oxidative stress protection in plants. Plant Development (John Innes Centre and Sainsbury Laboratory Annual Report), 41–42
Sadanandom A, Poghosyan Z, Fairbairn DJ, Murphy DJ (2000) Differential regulation of plastidial and cytosolic isoforms of peptide methionine sulfoxide reductase in Arabidopsis. Plant Pysiol 123: 255–263
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory, New York
Sánchez J, Nikolau BJ, Stump PK (1983) Reduction of N-acetyl methionine sulfoxide in plants. Plant Physiol 73: 619–623
Seymour GB, Taylor JE, Tucker GA (1993) Biochemistry of fruit ripening. London: Chapman and Hall.
Smith DB, Johnson KS (1988) Single step purification of polypeptides expressed in *E. coli* as fusion with glutation-S-transferase. Gene 67: 31–40
Speirs J, Longhurdt T (1993) RNA extraction and fractionation. In: Dey PM, Harborne JM (eds) Methods in Plant Biochemistry. pp 10, 1–33
Trainotti L, Spolaore S, Pavanello A, Baldan B, Casadoro G (1999) A novel E-type endo β-1,4-glucanase with a putative cellulose-binding domain is highly expressed in ripening strawberry fruits. Plant Mol Biol 40: 323–332
Varma SD, Kumar S, Richards RD (1979) Light-induced damage to ocular lens cation pump: prevention by vitamin C. Proc Natl Acad Sci USA 76: 3504–3506
Vogt W (1995) Oxidation of methionyl residues in proteins: tools, targets and reversal. Fre Radic Biol Med 18: 93–105
Yamashita I, Iino K, Nemoto Y, Yoshikawa S (1977) Studies on flavor development in strawberries. J Agric Food Chem 25: 1165–1168
Yamashita I, Nemoto Y, Yoshikawa S (1976) Formation of volatiles alcohols and esters from aldehydes in strawberries. Phytochemistry 15: 1633–1637
Zhao J, Zuo K, Wang J, Cao Y, Zhang L, Tang K (2003) cDNA cloning and characterization of a cotton peptide methionine sulfoxide reductase (cMsrA). DNA Seq 14: 303–310

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