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The cystathionine-γ-synthase gene involved in methionine biosynthesis is highly expressed and auxin-repressed during wild strawberry (*Fragaria vesca* L.) fruit ripening

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Abstract Differential screening of a cDNA library from ripe wild-strawberry *(Fragaria vesca)* receptacles allowed the isolation of a cDNA clone for an mRNA which accumulated to high levels in ripe strawberry receptacles. Sequence analysis of this cDNA (1940 bp) and the corresponding gene (4330 bp) indicated the presence of an ORF coding for a predicted protein of 545 amino acids (MWt 58.3 kDa) showing 91% identity with an *Arabidopsis thaliana* cystathionine γ-synthase (CGS), an enzyme involved in methionine biosynthesis. Up to now, CGS activity was reported only in leaves from various plants and in microorganisms. In this study, a much stronger expression was observed in receptacles from strawberries compared to other organs (leaves, flowers, etc.). Accumulation of CGS transcripts in receptacles of both wild and commercial strawberries was highly correlated with the progress of ripening. Immunoblot experiments with an *A. thaliana* CGS antibody demonstrated a similar profile of accumulation for CGS protein and transcripts. Southern-blot analysis of genomic DNA indicated that the CGS protein was encoded by a single gene in *Fragaria* species and in some climacteric fruits, e.g. peach and apricot. In addition to the ripening-related genes identified in strawberries, the treatment of receptacles (des-achenated or not) with the auxin NAA significantly reduced CGS gene expression.

Key words Gene regulation · Ripening-related protein · Non-climatic fruit

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Introduction

Ripening is a complex developmental process which affects physical, biochemical and sensory characteristics of fruits. Dramatical changes occur in firmness, color, taste and aroma development. Despite important differences in the hormonal regulation of the process, modifications at the genetic and enzymatic levels are similar in both climacteric (tomato, avocado, kiwi) and non-climacteric (strawberry, grape, cherry) fruits. Qualitative and quantitative changes in the mRNA population occurring during fruit development were documented in numerous species leading to the identification of ripening-related genes. Over the last decade, there have been considerable advances in understanding the function of such genes in climacteric fruits (Gray et al. 1994) and about their regulation by the plant hormone ethylene (Lelièvre et al. 1997). More recently, attention has been focused on non-climacteric fruits such as orange (Alonso et al. 1995), cherry (Fils-Lycaon et al. 1996) and strawberry. Considerable research has been done on genes highly expressed during strawberry ripening (Wilkinson et al. 1995; Medina-Escobar et al. 1997a; Manning 1998; Nam et al. 1999) and about their regulation by auxin which plays a key role in the control of the growth and ripening of strawberry receptacles (Nitsch 1950; Given et al. 1988). Five genes regulated by auxin were reported in strawberry species (Reddy and Poovaiah 1990; Reddy et al. 1990; Medina-Escobar et al. 1997b; Nam et al. 1999). Currently, molecular mechanisms and determinants which govern the growth of non-climacteric fruits remain poorly understood. To obtain additional information on these processes, our strategy consists in: (1) the isolation of highly and/or specifically expressed genes in non-climacteric fruit, (2) the identification of their function, and (3) their mode of regulation using a genetic transformation approach. In this respect, wild strawberry (*Fragaria vesca* L) is a convenient model since it has a small diploid genome and a short reproductive cycle. Moreover, its transformation and regeneration in vitro have already been reported (El Mansouri et al. 1996). A differ-

ential screening of a mature wild strawberry receptacle cDNA library allowed the isolation of genes undergoing an alteration in their expression pattern during strawberry development (Nam et al. 1999). A cDNA was isolated whose expression was highly induced during fruit ripening. In the present paper, we report the characterization of this cDNA (EMBL accession number AJ001451) and its corresponding gene (EMBL accession number Y17185) which encodes cystathionine γ-synthase (CGS), an enzyme involved in methionine biosynthesis. For the first time, it is reported that this gene is highly induced in fruits such as strawberry compared to the basal expression in others organs. The corresponding gene expression is down-regulated by auxins and seems to be correlated with the progress of ripening. As ripening processes are differentially regulated in climacteric and non-climacteric fruits, CGS gene expression was also analyzed in various fruit species whether climacteric or not.

Materials and methods

Plant material

Commercial (*Fragaria x ananassa* Duch. cv Pajaro) and wild strawberries (*F. vesca* cv Reine des Vallées) were grown in a greenhouse and daily irrigated. Strawberry fruits were collected at seven different stages of development. Different tissues of wild strawberry plants were sampled, such as adult and young leaves, flowers, roots and stems. The other fruits, peach (*Prunus persica)* and apricot *(Prunus armeniaca)*, at a commercial maturity stage (half-ripe) were also selected. These different fruits were separately frozen in liquid nitrogen without seeds (achenes, stones), ground to a fine powder and stored at –80°C for subsequent experiments.

Hormone treatment

Wild strawberries were collected at the breaker stage and treated with 1 µM of auxin NAA. The hormone solution (1% DMSO, 0.01% Tween 20, MS salts, hormone, pH 7) were sprayed over the fruit surface and fruits were harvested during 24 h with the pedicel dipping in the same solution. One set of fruits was des-achenated using the tip of a scalpel blade and treated with NAA solution as described for total fruits. Controls were carried out on total or des-achenated fruits sprayed with a solution without hormone. After 24 h of treatment, fruits were frozen without achenes, ground to a fine powder and stored at –80°C for Northern-blot experiments.

5´ Race-PCR

The initial cDNA clone was obtained from a differential screening of a mature wild strawberry (Nam et al. 1999). The insert of 711 bp was used as a probe (CGS probe) for subsequent hybridization experiments. The 5´ end of CGS cDNA was obtained by 5´ RACE-PCR (Boehringer Mannheim) according to the manufacturer's recommendations. Poly(A)+ RNA was obtained from receptacles of wild strawberry at the Ripe stage. The first-strand cDNA was synthesized using the cDNA-specific primer SP1 (5´-AGATTCCGCTCCTTCAAGAG-3´ at position 1387–1406 in Fig. 1). After degradation of the mRNA template, a homopolymeric A-tail was added to the 3´ end of the cDNA. PCR-amplification of this template was performed with an oligo dT-anchor primer (5´-GACCACGCGTATCGATGTCGAC$(T)_{16}$-3´) and another cDNA-specific primer SP2 (5´-CTCAAGAACCACGGTAGTTG-3´ at position 1246–1266 in Fig. 1). The resulting cDNA was further amplified by a second PCR using a nested specific primer SP3 (5´-CATCAGTCACTATACCGCGA-3´ at position 970–990 in Fig. 1) and a PCR anchor primer (5´-GACCACGCGTATCGATGTCGAC-3´). The PCR products were cloned in the pGEM-T vector (pGEM-T Vector System, Promega) at the *Eco*RI sites and submitted to nucleotide sequencing.

Inverse PCR

Genomic DNA was digested with an appropriate restriction enzyme, extracted by phenol/chloroform, precipitated by ethanol and re-suspended in TE buffer at a concentration of 0.5°µg/µl. DNA was diluted to 1–10 µg/ml in ligase buffer with 60 units of ligase (concentrated ligase from Promega) and incubated at 4°C over night. After ligation, DNA was extracted by phenol/chloroform, precipitated by ethanol and re-suspended in TE buffer at a concentration of 100 ng/µl. PCR-amplification was performed as normal, the annealing temperature of which depended on the size (around 30 bp) and composition of the selected primers. The PCR-amplification performed on the DNA digested by *Hin*dIII and ligated, used the iPCR1 primer (5´-TTGGTGGTTGTGAGAGCATCGTGGATCA-3´) and the iPCR2 primer (5´-AACCAGGATAATAGACGTGCGCCACCTT-3´), whereas, the PCR-amplification performed on the DNA digested by *Eco*RV and ligated, used iPCR3 primer (5´-TCACCTCCTCCTCCTCCTCCTCCACCTACT-3´) and iPCR4 primer (5´-CCATGGTGGTGAGAGTGAGGAAGATTGATG-3´). The PCR program employed for DNA-amplification in this work was adapted from the Boehringer protocol for the "Expand Long Template" *Taq* enzyme. The amplified fragment was cloned in pGEM-T vector (Promega) and submitted to sequencing up to the restriction-enzyme site.

Nucleotide sequencing and analysis

Plasmid DNA minipreparations were carried out according to Sambrook et al. (1989). DNA sequencing was carried out with a Sequenase Version 2.0 DNA Sequencing Kit (Amersham) using T3 and T7 universal primers or specific internal primers, labelled with α-^{33}PdATP. Sequences were compared to EMBL and GenBank databases using the BLAST (Basic Local Alignment Search Tool) program.

Radioactive DNA probes

The CGS cDNA of 711 bp from the cDNA library was used as a probe (CGS probe) for Southern- and Northern-blot experiments. The corresponding plasmid was digested by *Xho*I and *Eco*RI restriction enzymes in order to release the insert, and was purified from the vector on an agarose gel. Recovery and elution of the insert (711 bp indicated in Fig. 1) was carried out using a resin-based purification system. (Wizard PCR Preps, Promega). The insert was radioactively labelled with α-^{32}P dCTP by the random labelling technique ("Ready to Go", Pharmacia). A potato 25 S rDNA fragment containing a coding region was used as the control probe for rRNA quantity.

Northern-blot analysis

Total RNA from different fruits and wild strawberry tissues were isolated according to Manning's protocol (Manning 1991). Fifty micrograms of total RNA were denatured and fractionated on denaturing gels (1.2% agarose containing 7.5% formaldehyde and 1×MOPS). The corresponding gel was transferred to a nylon membrane (NytranPlus, Schleicher and Schuell) using 20×SSC as the transfer buffer. The membrane was baked at 80°C for 2 h, pre-hybridized for 2 h at 40°C with 50% formamide, 6×SSC, 1×SDS, 2×Denhardt's solution and 100 µg/ml of salmon-sperm DNA. Membrane hybridization was carried out in the same solution with a heat-denatured probe for at least 16 h. Membranes were washed twice at room temperature in 2×SSC for 10 min, three times at 65°C in 2×SSC–1×SDS for 20 min and then exposed to X-ray film.

Southern-blot analysis

Five grams of young leaves were frozen in liquid nitrogen, ground to a fine powder and re-suspended in 25 ml of cold extraction buffer (sorbitol 0.35 M, Tris 0.1 M, EDTA 5 mM, β-mercaptoethanol 1%, pH 7.5) and filtered through miracloth paper. The filtrate was centrifuged at 1000 g for 15 min at 4°C. The pellet was re-suspended in 2.5 ml of extraction buffer to which were added 2.5 ml of nuclei lysis buffer (Tris 200 mM, EDTA 50 mM, NaCl 2 M, CTAB 2%) and 1 ml of sarcosyl 5%. This mixture was agitated at 65°C for 30 min. An equal volume of chloroform/isoamyl alcohol (24/1) was added before centrifugation at 3500 g at room temperature for 25 min. The DNA was precipitated in the aqueous phase by adding 2/3 vol of isopropanol and centrifuged at 12,000 g, at 4°C for 20 min. The pellet was washed with 5 ml of ethanol 70% and then re-suspended in 300 µl of Tris-EDTA pH 8. The DNA concentration was determined by nucleic acid absorption at 260 nm and used for Southern-Blot analysis. Fifty micrograms of genomic DNA from wild strawberry were digested with different restriction enzymes such as *Eco*RI, *Eco*RV, *Hin*dIII, *Xho*I. Fifty micrograms of DNA from different three species, commercial strawberry, peach and apricot, were digested by *Eco*RV. The different digestions were fractionated on a 0.8% agarose gel and transferred to nylon membrane (Nytran, Schleicher and Schuell) using 0.2% M NaOH as the transfer buffer. Membranes were baked, pre-hybridized and hybridized with the CGS probe under the same conditions as for the Northern Blot.

Western-blot analysis

The production of antiserum raised against *Arabidopsis thaliana* CGS and the immunoblotting reaction were carried out by J. Kim and T. Leustek. Polyclonal antibodies were produced using purified recombinant CGS with 6× His *Taq*. An initial injection consisted of a 400-µl emulsified PBS solution containing 1 mg of the recombinant protein injected into rabbits in order to produce the anti-CGS antibodies. The rabbits were injected subcutaneously at 1-month intervals with 1 mg of the recombinant CGS. Seven days after the fourth boost, a 20-ml bleed was collected from the rabbit and used directly in immunoblot analysis. Immunoblotting against CGS was carried out in order to test the specificity of the immune antisera with the pre-immune sera as control. Total proteins of strawberries were extracted as described by Meyer et al. (1988). The protein concentration was determined by the protein-assay dye reagent (Bio-Rad). Protein samples (10 µg) were run on an SDS-PAGE gel containing 7.5% (w/v) of polyacrylamide and blotted onto an Immobilon-P membrane (Millipore Corp.) at 4°C for 1 h by using a transfer buffer (25 mM Tris and 250 mM glycine). Membranes were blocked with 10% ovalbumin (w/v) in PBST [0.1 M Phosphate buffer pH 7.4, 15 mM NaCl, 3 mM KCl, 0.2% (v/v) Tween-20] overnight. The membrane was reacted with antibodies raised against *Arabidopsis* CGS with a 1:2000 dilution of PBST for 1 h and then washed 3-times with PBST for 3 h. The membrane was then probed with an anti-rabbit secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase – Sigma) diluted 1:8000 in PBST for 30 min. After three washing steps in PBST for 2 h each, the membranes were probed with a chemiluminescent reagent (Dupont NEN) and exposed to film for periods ranging from 1 to 20 min.

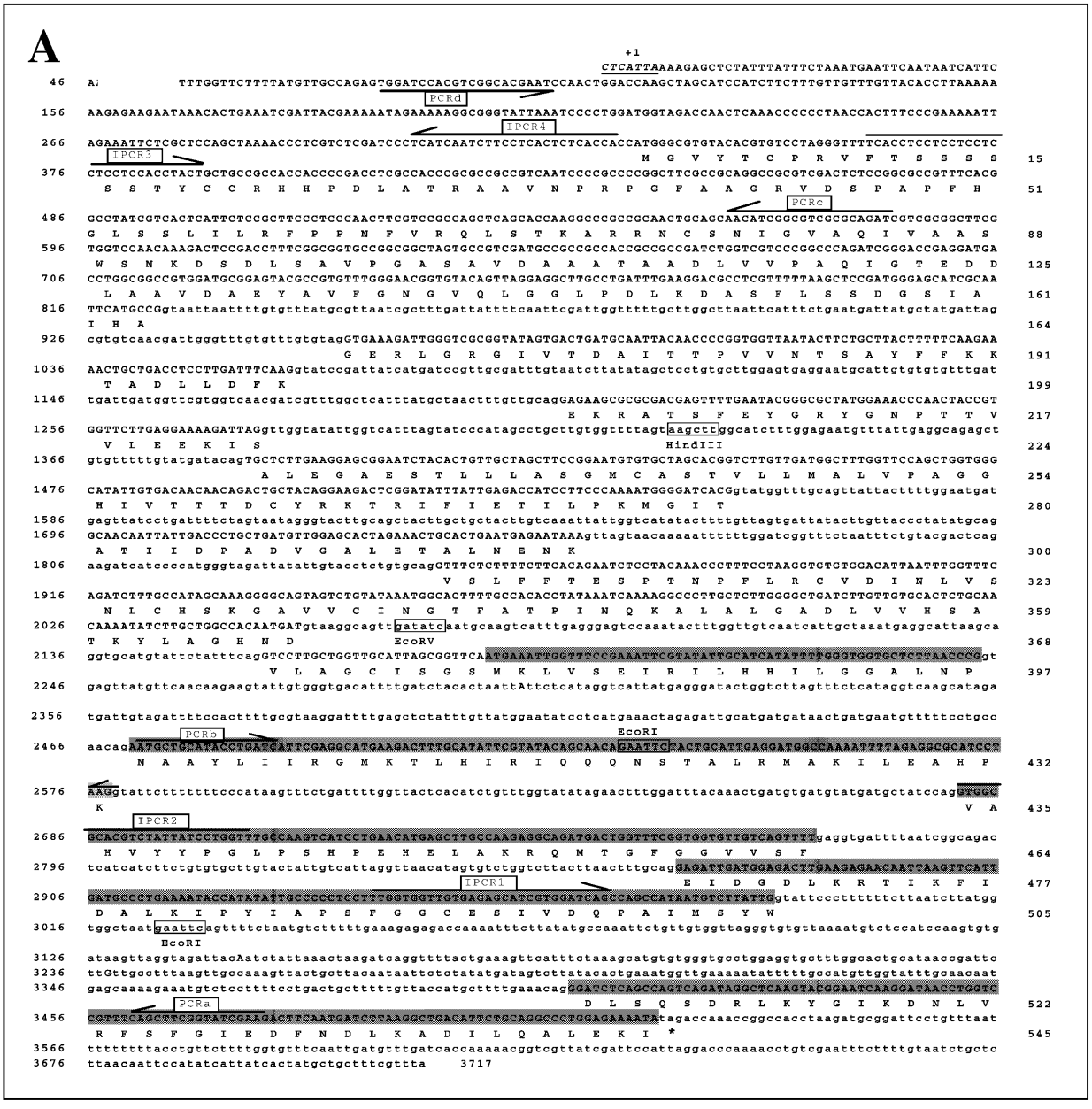
Results

Identification of wild strawberry CGS cDNA and its corresponding gene

A cDNA library constructed from mature wild strawberry receptacles was differentially screened in order to isolate clones corresponding to genes preferentially expressed in mature fruits (Nam et al. 1999). Among these, a cDNA clone was selected and analyzed at both the nucleotide and the deduced amino-acid level. The cDNA contained 711 bp with a partial open reading frame (ORF) of 168 amino acids. The 5´-flanking sequence was determined using RACE-PCR. The entire cDNA sequence was 1940 bp (accession number AJ001451) with an ORF of 545 amino acids (Fig. 1) and a predicted molecular weight of 58.3 kDa. Database comparisons showed a high homology with an *A. thaliana* gene encoding a cystathionine γ-synthase, an enzyme involved in methionine biosynthesis (Le Guen et al. 1994; Kim and Leustek 1996). This protein showed 91% identity over 320 amino acids (Fig. 2) and 77% over 1180 nucleotides. Lower homologies were found with proteins belonging to the methionine pathway in microorganisms (Fig. 2): about 60% identity over 150 amino acids with *Escherichia coli* metB (Duchange et al. 1983) and *Pseudomonas putida* L-methionine γ-lyase (Inoue et al. 1995). As well as these enzymes, the wild strawberry CGS protein presented a signature sequence for a pyridoxal-5´-phosphate binding site (LGADLVVHSATKY) located at position 350–362 (Fig. 2) and the lysine residue (position 361) which was shown to bind covalently to the cofactor. Compared to bacterial proteins, the wild strawberry CGS gene encoded a protein with a large amino-terminal sequence that resembled looked a transit peptide for localization to plastids. The CGS gene was isolated by inverse PCR. This method is based on the amplification of flanking opposite genomic sequences with primers defined from the corresponding cDNA. The sequence of the CGS gene was determined from two overlapping iPCR fragments and two conventional PCR-amplifications (Fig. 1B). Using this strategy, the complete 4330-bp gene sequence was obtained corresponding to 3720 bp of the gene and 610 bp of the promoter region. The genomic sequence presented ten introns flanked by the consensus nucleotides defined by Brown (1986): these started as GTA nucleotides, with some variations G(T/A)(A/T/G), and ended by CAG (TAG for intron 10). The localization of introns and exons were well-conserved between wild strawberry and *A. thaliana* (the only CGS genes isolated from plants) while their length could change, as illustrated by intron 10 which was 415-bp or 105-bp long respectively (Fig. 1B).

Gene organization in wild strawberry and other fruit species

The copy number of the CGS gene was estimated by Southern-blot analysis in wild strawberry and in other fruit species. Genomic DNA from wild strawberry was digested by restriction enzymes with recognition sites in the cDNA, such as *Eco*RI (indicated in Fig. 1), or without recognition sites, such as *Eco*RV, *Hin*dIII and *Xho*I. In parallel, genomic DNAs from plants of the same botanical family, i.e. commercial strawberry (*Fragaria ananassa)*, or from apricot (*P. armeniaca)* and peach (*P. persica*), were digested by the *Eco*RV restriction en-


A
+1
CTCATTAAAAGAGCTCTATTTATTTCTAAATGAATTCAATAATCATTC
PCRd
IPCR4
IPCR3
M G V Y T C P R V F T S S S S
S S T Y C C R H H P D L A T R A A V N P R P G F A A G R V D S P A P F H
PCRc
G L S S L I L R F P P N F V R Q L S T K A R R N C S N I G V A Q I V A A S
W S N K D S D L S A V P G A S A V D A A A T A A D L V V P A Q I G T E D D
L A A V D A E Y A V F G N G V Q L G G L P D L K D A S F L S S D G S I A
I H A
G E R L G R G I V T D A I T T P V V N T S A Y F F K K
T A D L L D F K
E K R A T S F E Y G R Y G N P T T V
V L E E K I S
HindIII
A L E G A E S T L L L A S G M C A S T V L L M A L V P A G G
H I V T T T D C Y R K T R I F I E T I L P K M G I T
V S L F F T E S P T N P F L R C V D I N L V S
N L C H S K G A V V C I N G T F A T P I N Q K A L A L G A D L V V H S A
T K Y L A G H N D
EcoRV
V L A G C I S G S M K L V S E I R I L H H I L G G A L N P
PCRb
EcoRI
N A A Y L I I R G M K T L H I R I Q Q Q N S T A L R M A K I L E A H P
K
V A
IPCR2
H V Y Y P G L P S H P E H E L A K R Q M T G F G G V V S F
E I D G D L K R T I K F I
IPCR1
D A L K I P Y I A P S F G G C E S I V D Q P A I M S Y W
EcoRI
PCRa
D L S Q S D R L K Y G I K D N L V
R F S F G I E D F N D L K A D I L Q A L E K I *
-300bp
+1
ATG
+3200bp
A. thaliana
E1
E2
E3
E4
E5
E6
E7
E8
E9
E10
E11
I1
I2
I3
I4
I5
I6
I7
I8
I9
I10
-610bp
+1
+3720bp
F. vesca
EcoRV
iPCR4
EcoRV
EcoRI
EcoRI
iPCR3
PCRc
HindIII
iPCR2
HindIII
PCRd
iPCR1
PCRa
PCRb
250 bp

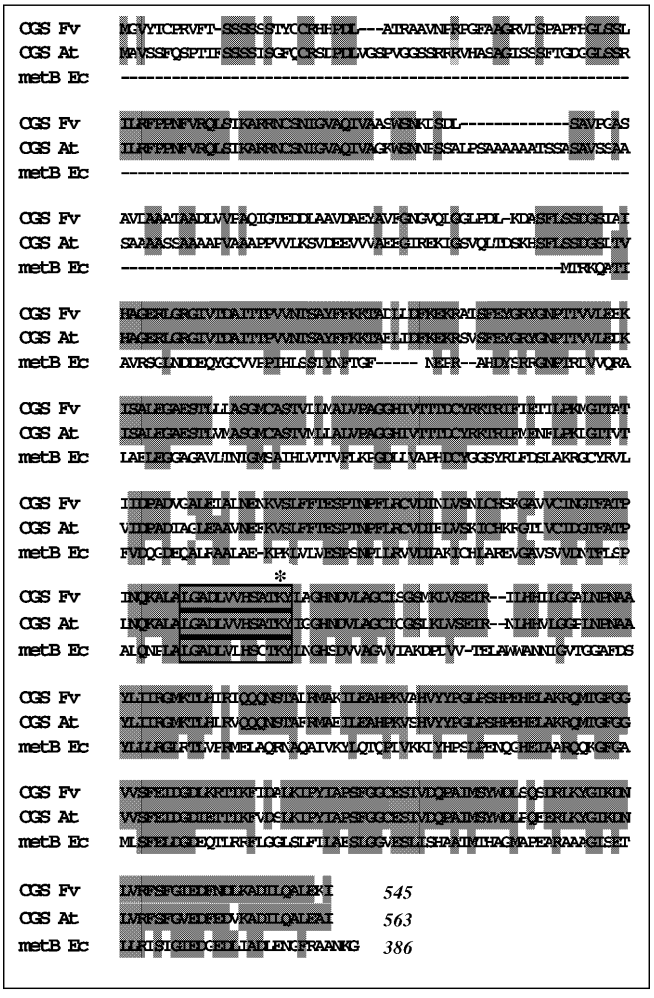


Fig. 2 Multiple alignment of the wild strawberry CGS protein (CGS Fv), *A. thaliana* cystathionine γ-synthase (*CGS At* – U43709) (Kim and Leustek 1996), and protein *metB* from *E. coli* (*metB Ec* – P00395) (Duchange et al. 1983). The signature sequences for the pyridoxal phosphate binding site are *framed,* including the lysine residue (*K*) indicated by an *asterisk*

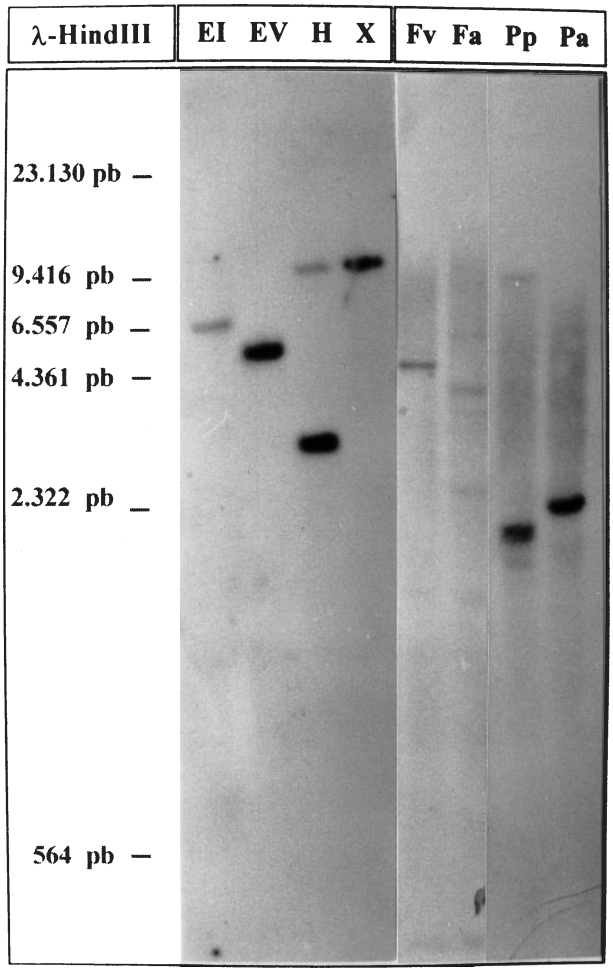


Fig. 3 Genomic organization of the CGS gene in wild strawberry and various plants. Genomic DNAs from wild strawberry were digested by *EI Eco*RI, *EV Eco*RV, *H Hin*dIII and *X Xho*I, while genomic DNA from various plants was digested by *Eco*RV. *Fv Fragaria vesca* (wild strawberry), *Fa Fragaria ananassa* (commercial strawberry), *Pa Prunus armeniaca* (apricot), *Pp Prunus persica* (peach)

zyme. The corresponding digested DNAs were hybridized to the wild strawberry CGS insert probe. The Southern-blot pattern (Fig. 3) indicated a major band of 7 kbp and a minor band of 0.5 kbp as a result of an *Eco*RI digestion which confirmed the internal *Eco*RI site within the gene. Digestions using *Eco*RV and *Xho*I led to a single band whereas *Hin*dIII digestion yielded two bands one of which was major. According to the results of Southern hybridization and the gene map, it appeared that the CGS protein was encoded by a single gene in wild strawberry. Similar results were obtained for heterozygous species: only one hybridizing band was detected by Southern blotting for the diploid peach and apricot, whereas in the octoploid commercial strawberry at least four bands were observed.

◄ **Fig. 1 A** Nucleotide and deduced amino-acid sequences of the cystathionine γ-synthase of the wild strawberry gene and cDNA. *Block capitals* indicate the coding sequences corresponding to the cDNA and exons from the CGS gene. *Lower cases* are the non-coding sequences corresponding to ten introns of the gene. The stop codon is marked with an *asterisk*. The *grey coding sequence* indicates the 711-bp CGS insert isolated from cDNA library and subsequently used as a CGS probe. The *iPCR 1–4* are divergent primers used in inverse PCR whereas *PCR a to d* are convergent primers used in PCR. **B** Gene isolation strategy and structure of the wild strawberry CGS gene versus *A. thaliana*. Exons (*E*) are indicated by *solid boxes* bound by introns (*I*). The iPCR technique applied for isolation of the wild strawberry CGS gene is schematically represented by the location of the primers employed

Regulation of CGS gene expression in strawberry and other fruits

With a view to determining a the expression pattern of the CGS gene during fruit development, seven developmental stages of wild and commercial strawberries were characterized from fruit set to senescence, according to weight, color, sugar and organic acid contents (data not shown). Total RNA was isolated and the corresponding Northern

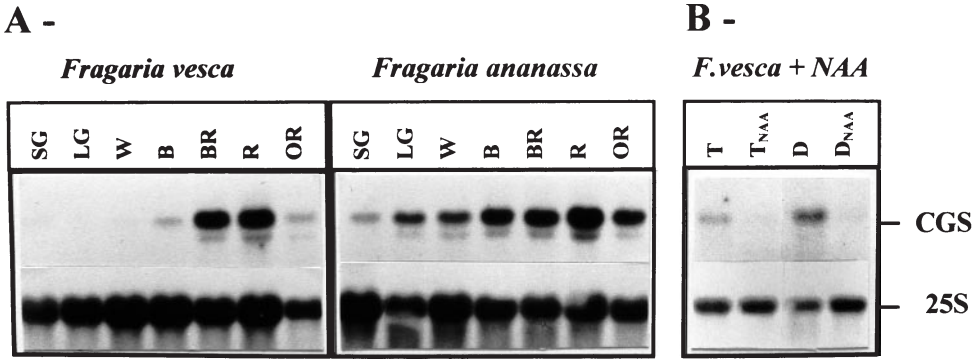


Fig. 4 **A** Expression of the CGS gene during wild and commercial strawberry development. *SG* small green, *LG* large green, *W* white, *B* breaker, *BR* breaker-ripe, *R* ripe, *OR* over-ripe. **B** Effect of auxin on CGS gene expression: hormone treatments were applied to wild strawberry receptacles at the breaker stage. *T* total fruit, *T+NAA* total fruit treated with 1 µM of NAA, *D* des-achenated fruit, *D+NAA* des-achenated fruit treated with 1 µM of NAA. Hybridizations were carried out with CGS and 25 S rDNA probes

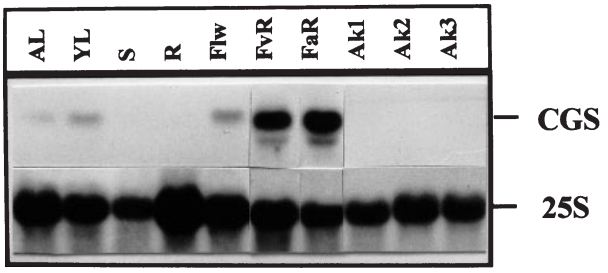


Fig. 5 Detection of CGS mRNA in various plant organs from wild strawberry. Total RNAs were hybridized to CGS and 25 S rDNA probes. Three developmental stages of achenes (*Ak 1–3*), *AL* adult leaves, *YL* young leaves, *S* stem, *R* roots, *Flw* flowers, *FvR F. vesca* ripe receptacles, *FaR F. ananassa* ripe receptacles

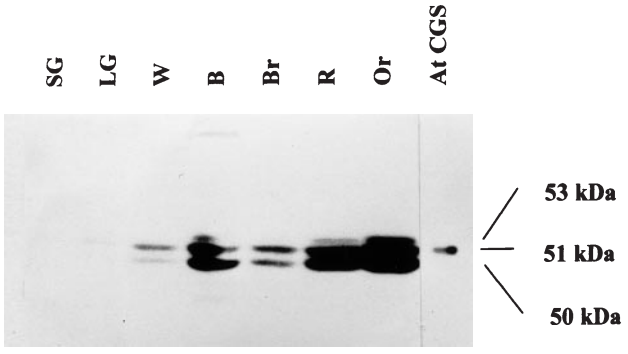


Fig. 6 Detection of CGS polypeptides in wild strawberry receptacles. Pure *A. thaliana* CGS and proteins from different developmental stages of wild strawberry receptacles were treated with antibodies raised against *Arabidopsis* CGS. *SG* small green, *LG* large green, *W* white, *B* breaker, *BR* breaker-ripe, *R* ripe, *OR* over-ripe

blots hybridized to CGS or 25 S rDNA probes. This last hybridization gave a good evaluation of total RNA quantity. A hybridization carried out with the CGS probe yielded a signal corresponding to a 2000 base-length transcript (Fig. 4A). In both cultivars, CGS transcripts gradually increased in late developmental stages of receptacles where ripening processes occurred. While these accumulated transcripts significantly from the Breaker stage in wild strawberries, they were detectable from the beginning of fruit development in commercial strawberries. As auxin regulates strawberry development by promoting growth and inhibiting ripening, the hormone effect was analyzed on the CGS gene. Hormone experiments were conducted on de-sachenated receptacles or entire fruits since auxin was concentrated in the achenes. A set of samples was treated for 24 h with 1 µM of NAA auxin whereas another set was used as a reference. Northern-blot results showed that CGS gene-expression was significantly reduced by the addition of the hormone on both treated fruits (Fig. 4B). Furthermore, a higher expression was observed in de-sachenated fruits compared to total fruits. This implied that CGS gene expression is significantly down-regulated by endogenous auxin in achenes or by the application of exogenous auxin.

In order to assess the specificity of CGS gene-expression to fruit ripening, CGS gene-expression was also analyzed in other wild strawberry organs such as: (1) achenes at different maturation stages, (2) actively growing organs such as flower buds and young leaves, and (3) quiescent organs such as adult leaves, roots and stem. A weak accumulation of transcripts was detected in flowers and leaves independently of their developmental stages (Fig. 5). After a long exposure of the RNA-blot, a basal CGS gene-expression was revealed in all tested organs. As greater CGS gene-expression was displayed in fruits than in other plant organs; the expression was also analyzed in some other fruits such as peach and apricot, but no accumulation of similar transcript was observed (data not shown).

CGS protein accumulation during strawberry development

Detection of the corresponding protein was carried out in order to assess the ability of CGS transcripts to be translated. Western-blot analysis was performed on proteins extracted from strawberry receptacles which were hybridized with an antibody raised against *A. thaliana* CGS. Two major bands of approximately 50 and 51 kDa, as well as a minor one of 53 kDa, cross-reacted with the

antibody (Fig. 6) and were also detected in commercial strawberries (data not shown). The time-course of CGS protein accumulation is closely correlated with the accumulation of the transcripts. Analysis of purified CGS from spinach (Ravanel et al. 1995) and *A. thaliana* (Ravanel et al. 1998) chloroplasts revealed that native CGS was a homotetramer composed of 53-kDa monomers, as two subunits of unequal abundance of 50 and 53 kDa were displayed. Since these two polypeptides were identical at their N-termini, a proteolytic degradation at the COOH terminus of the 53-kDa subunit was suggested and attributable to the purification process (Ravanel et al. 1998). According to these data, the three different polypeptides displayed in strawberry could result from several proteolytic cleavages of the 53-kDa subunit during processing with acidic strawberry fruits, but in vivo post-translational cleavages or covalent modifications of the subunit are still not excluded.

Discussion

A cDNA which is highly expressed during wild strawberry fruit ripening was isolated and characterized for its potential functions and regulation in the ripening processes. Its translational product showed high homology (91%) with *A. thaliana* cystathionine γ-synthase (Kim and Leustek 1996) and lower homology (around 60%) with bacterial proteins of the methionine pathway. Cystathionine γ-synthase is the first enzyme involved in methionine biosynthesis which catalyses the condensation of homoserine derivative forms with cysteine, forming cystathionine as an intermediate compound. Up to now, CGS activity was only detected in leaves (Wallsgrove et al. 1983, Ravanel et al. 1995, Ravanel 1997) and seedlings (Kreft et al. 1994). The present work shows that in some soft-fruit plants, such as strawberry, CGS transcripts and proteins were more highly accumulated in fruits than in other organs. Furthermore, it has been previously claimed that CGS protein was only present in chloroplasts (Ravanel et al. 1995; Kim and Leustek 1996); but the presence of the proteins in red ripe strawberries indicates that this enzyme is also found in chromoplasts. The physiological substrate of CGS is preferentially O-phospho-L-homoserine (OPH) in plants (Datko et al. 1974) and O-succinylhomoserine or O-acetyl-L-homoserine in bacteria (Duchange et al. 1983). This OPH substrate is partitioned between two major metabolic pathways, threonine and methionine biosynthesis. This implies a regulatory role for each first metabolic enzyme, and particularly for CGS in the methionine pathway (Giovanelli 1990). Actually only this methionine pathway was previously known and a single CGS gene was displayed in plants (Kim and Leustek 1996). Thus, the presence of such a protein in strawberry fruits implied a great induction of methionine biosynthesis during fruit ripening. This sulfur amino acid not only plays an essential role as a metabolic component in protein biosynthesis but also as a regulatory component in its derivative form, S-adenosyl methionine (SAM), which acts as a methyl donor and a precursor of regulatory compounds such as ethylene and polyamines (Giovanelli 1990). The very low level of CGS transcripts in other plant tissues suggests a basal methionine synthesis, certainly for metabolic pathways, whereas the high methionine synthesis during strawberry ripening might be correlated with regulatory events considering the dramatic changes that occur during this developmental phase. If in climacteric fruits (tomato, melon, avocado), ethylene is autocatalytically synthesized and acts as the major regulator of the ripening processes, its production decreases during the development of non-climacteric fruits (strawberry, grape). Perkins-Veazie et al. (1995) have shown that ethylene production in strawberry fruits decreased sharply as the fruit ripens. This suggests that ethylene synthesis is not the major mode of methionine metabolism in strawberry fruits. Similarly, polyamines are known for their regulatory functions in numerous developmental processes, including fruit ripening. A recent paper (Martinez-Madrid et al. 1996) reported low levels of free polyamines during the rapid ripening of tomato whereas the development of non-climacteric citrus fruits (mandarin and orange) is associated with an increase in free polyamine content. Nevertheless, these results have not definitively established a correlation between the rate of fruit ripening and polyamine content. Thus, a relationship between an increase of polyamine synthesis and strawberry ripening could partially explain the increase in CGS activity and methionine synthesis. Other regulatory mechanisms such as the methylation of macromolecules (proteins, DNA) could also participate in the important cellular changes which lead the fruit to ripen. Considering these different methionine pathways, and at this point of the investigation, it remains speculative to predict if one of these mechanisms is predominant or if they act in synergy to promote the ripening process of strawberries.

As well as ethylene and polyamines, some other hormones such as abscisic acid and auxin participate in the regulation of the ripening processes. In strawberry fruits, auxin is considered as the primary hormone regulating fruit development by promoting receptacle expansion and inhibiting ripening (Nitsch 1950; Manning 1994). The removal of achenes, in which auxin is concentrated, induced ripening of the receptacle and CGS gene expression, whereas application of auxin on de-sachenated receptacles delayed ripening and repressed CGS gene-expression. Thus, the CGS gene is down-regulated by auxin as only a few ripening-related genes have yet been reported in strawberry, such as a pectate lyase (Medina-Escobar et al. 1997b) and an endo-1,4-β-glucanase (Harpster et al. 1998) which both directly participate in the ripening process, inducing fruit softening.

The correlation between the ripening-related and auxin regulation of CGS gene expression strongly supports a role for protein in the ripening processes of strawberries. Nevertheless, this ripening associated function in strawberry cannot be extended to other fruits since no CGS

gene-expression has been displayed in fruits such as apricot and peach. More information relative to the CGS function in ripening will be become available by genetic transformation of wild strawberry with the antisense gene. Moreover, it would be of interest to determine the up-regulation of such a gene in order to improve methionine biosynthesis in agronomic plants since this essential amino acid is necessary for human nutrition.

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