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cDNA cloning and expression of cysteine synthase B localized in chloroplasts of *Spinacia oleracea*

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The cDNA clones for cysteine synthase B, which is localized in chloroplasts of *Spinacia oleracea* L., were isolated by screening a library with synthetic oligonucleotides encoding a partial peptide sequence of the purified protein. Nucleotide sequence analysis revealed an open reading frame encoding a polypeptide of 383 amino acids containing a putative transit peptide of 52 amino acids. A bacterial expression vector of the cDNA clone could genetically complement an *Escherichia coli* auxotroph lacking cysteine synthase and could produce the functionally active and immuno-reactive cysteine synthase in *E. coli* RNA blot hybridization suggested that the transcripts were primarily accumulated in leaves of spinach.

Cysteine synthase; cDNA cloning; Signal peptide, Sulfur assimilation; Genetic complementation, *Spinacia oleracea*

1. INTRODUCTION

Inorganic sulfur is assimilated to cysteine through the cysteine biosynthetic pathway in plants and bacteria, whereas animals do not have the assimilation pathway of inorganic sulfur and require a dietary source of methionine for sulfur metabolism [1]. Cysteine is the principal starting metabolite for the synthesis of other sulfur-containing compounds such as methionine, glutathione and some secondary S-compounds. The crucial fixation step of inorganic sulfide to cysteine, the first sulfur containing organic compound, is catalyzed by cysteine synthase (CSase) [*O*-acetyl-L-serine (thiol)-lyase, *O*-acetyl-L-serine acetate-lyase (adding hydrogen sulfide), EC 4.2.99.8]. This pyridoxal phosphate-dependent enzyme catalyzes the formation of cysteine and acetic acid from *O*-acetylserine and hydrogen sulfide [2,3]. This enzyme is also responsible for the biosynthesis of some neuro-active heterocyclic β-substituted alanines [4], e.g. quisqualic acid in *Quisqualis indica*. The corresponding heterocyclic compounds are incorporated to the substituted alanine moiety instead of hydrogen sulfide to form these secondary non-protein amino acids.

In plant cells, there are three isoforms of CSase of different subcellular localizations. In spinach (*Spinacia oleracea* L.) green leaves [5], the two major activities are localized in cytoplasm and chloroplasts as CSase A and B, respectively. A minor activity is localized in mitochondria. Recently we have cloned and characterized cDNA encoding cytoplasmic CSase A from spinach leaves [6]. In this paper, we report the isolation of a cDNA clone encoding CSase B localized in spinach chloroplasts, the identification of cleavage site of transit peptide, and the results of expression study.

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The sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank data base (accession no. D14722).

Abbreviation. CSase, cysteine synthase

2. MATERIALS AND METHODS

2.1 *Microsequencing of peptide*

CSase B was purified from the leaves of spinach to apparent homogeneity on SDS-PAGE ([7], K. Saito et al., in preparation). The amino acid sequence of the internal peptide fragment (*V8-B7*) of the purified CSase B was determined after digestion with *Staphylococcus aureus* V8 protease as described [8]. The sequence of amino terminal (*V8-B0*) of CSase B was determined after concentrating the purified protein onto Immobilon P membrane (Millipore) for the sequencing reaction by a gas-phase protein sequencer according to [8].

2.2. *Screening of a cDNA library and DNA sequence analysis*

A cDNA library was constructed with λgt10 vector from poly (A)$^+$ RNA of 10-week-old leaves of *S. oleracea* cv. parade (Sakata Co., Yokohama, Japan) as described [6]. Approximately 1.6×10^5 non-amplified plaques were screened with two synthetic oligonucleotide probes encoding the internal peptide fragment *V8-B7*. Probe B71 encoding the entire sequence of *V8-B7* was a 53-mer in length, 3′- TGI TGI CCI GGI CTT TAI ACC CTT CTG TGI TTT CCI TTT CAI CTG TAI AAG CA -5′. Probe B72 encoding the partial sequence of *V8-B7* from Ile-6 to Ile-16 was a 32-mer in length, 3′- TAI ACC CTT CTG TGI TTT CCI TTT CAI CTG TA -5′. Duplicate filters (Hybond N+, Amersham) were obtained and each filter was hybridized with the two ^{32}P-labeled probes, respectively. The final washes on post-hybridization were performed in 5 × SSPE [9] and 0.1% SDS at 42°C.

Among the 20 isolated clones, two, designated as λCSB2 and λCSB11, possessing ca. 1.5-kb-length inserts were subcloned into M13mp18 for nucleotide sequencing. The DNA sequence was deter-

mined on both strands by the dideoxy method using a series of synthetic primers.

2.3. *Expression in a cysteine-auxotroph, E. coli NK3*

The 1.5-kb insert of λCSB2 was isolated by *Eco*RI digestion and cloned into the *Eco*RI site of pUC19 to yield pCSB2 with the sense orientation to the *lacZ* promoter. A cysteine-auxotroph, *E. coli* NK3 (ΔtrpE5 leu-6 thi hsdR hsdM$^+$ cysK cysM), was transformed with pCSB2. For genetic complementation of the cysteine requirement, the transformed *E. coli* was cultured on an M9 agar plate [9] supplemented with 0.02% leucine and tryptophan.

For Western blotting and enzyme assays, *E. coli* was grown in LB medium [9] containing carbenicillin (100 mg/l) for 2.5 h at 37°C; isopropyl β-D-thiogalactoside was added to 1 mM and the incubation continued for 1.5 h. The total soluble protein of *E. coli* was obtained as described [6]. Western blot and immunostaining analysis were carried out on an Immobilon P membrane using 3,3'-diaminobenzidine as the developer as reported [10]. The rabbit primary antibody against CSase A of spinach [6] was used at 1:200 dilution. The enzymatic activity of CSase was determined as described [4].

2.4. *Nucleic acid hybridization analysis*

For DNA hybridization analysis, total DNA (20 mg) of *S. oleracea* cv. parade was digested with restriction enzymes, separated on a 0.8% agarose gel, transferred to a Hybond N+ filter and then hybridized with the random-primer-labeled cDNA as a ^{32}P-probe as reported [11]. For RNA gel blots, total RNA was isolated from leaves and roots of 4- and 10-week-old spinach by the method reported [10]. Twenty μg of total RNA was denatured and separated in a formaldehyde agarose (1.2%) gel; followed by transfer to a Hybond N+ filter. Hybridization was carried out as described [6]. For both DNA and RNA blot hybridization, the final washes of the filters were in 0.1 × SSPE and 0.1% SDS at 65°C for 15 min.

3. RESULTS AND DISCUSSION

3.1. *Determination of amino acid sequences and isolation of cDNA clones encoding CSase B*

CSase B was purified from green leaves of spinach and proved to be localized in chloroplasts by immunostaining ([7], K. Saito et al., in preparation]. The amino acid sequences of N-terminal of the protein and of the internal fragment after digestion with *S. aureus* V8 protease were determined by microsequencing the peptide blotted onto a membrane filter (Fig. 1). On screening of the non-amplified library comprising 1.6×10^5 plaques with two synthetic oligonucleotides, 20 clones were isolated as doubly positive clones. Two clones, λCSB2 and λCSB11, with a ca. 1.5-kb-length insert were selected for further analysis.

3.2. *Sequence of CSase B cDNA*

Sequence determination of λCSB11 revealed the open reading frame of 1149 bp encoding 383 amino acids (Fig. 1). The calculated molecular mass of the encoded peptide was 40,636 Da. The amino acid sequence of fragment *V8-B7* was completely identical with that predicted from cDNA. The N-terminal sequence (*V8-B0*) of purified cysteine synthase B also showed identity with the predicted sequence from cDNA, except for two residues in *V8-B0*; Ser-55 in cDNA was Ala in the fragment *V8-B0* and Glu-69 in cDNA was absent in *V8-B0*. These results clearly indicated that the isolated cDNA clone encoded a polypeptide, pre-CSase B, possessing signal peptide of 52 amino acid residues at the N-terminal. This signal peptide is processed at the site between Lys-52 and Ala-53 to form the mature protein of 34,998 Da. The mismatching of amino acid residues between the sequence predicted from cDNA and the purified peptide can be due either to the microheterogeneity among cultivation varieties of spinach or to the presence of different transcripts from several copies of gene family.

The insert of λCSB2 had a shorter 5'-leader sequence of 10 bp and a different upstream position of poly (A) tail attachment. Additionally, A-95 in the sequence of λCSB11 was changed to C in λCSB2 to cause equivalent amino acid replacement of Ile in λCSB11 to Leu in λCSB2. Two putative signal sequences of poly (A) addition were identified at 22 bp and 25 bp upstream of the poly (A) tails of λCSB11 and λCSB2, respectively.

Very recently, Rolland et al. [12] reported a cDNA sequence of *S. oleracea* similar to our present clones. However, the sequence determined from one clone was shorter than the one presented in this paper and showed some mismatches leading to 3 amino acid changes; Ile-12 → Leu, Ala-334 → Arg, Ala-335 → Arg. The change of Ile → Leu was also observed in our sequences as mentioned. However, as shown in Fig. 2, Ala-334 and 335 are highly conserved among the sequences of CSases.

3.3. *Comparison of the deduced amino acid sequences with other CSases*

The deduced amino acid sequence of λCSB11 showed the homology to those of CSase localized in chromoplasts of *Capsicum annuum* (75%) [13], of spinach CSase A localized in cytoplasm (73%) [6], of wheat CSase A (70%) [14], of *E. coli* and *Salmonella typhimurium* CSase encoded on *cysK* (54% and 53%, respectively) [15,16], and *E. coli* CSase encoded on *cysM* (40%) [17]. The multiple alignment of predicted amino acid sequences indicated that the sequences after the cleavage position at 52–53 are homologous in all seven proteins (Fig. 2), and, therefore, this region is the catalytically functional peptide.

The sequence of amino acid positions 1–52 of spinach CSase B showed the general features of transit peptide for transport of protein to chloroplasts in all aspects postulated by the previous studies [18]: (i) it starts with Met-Ala; (ii) it is rich (10 out of 52 residues) in hydroxylated amino acids, Ser and Thr; (iii) it is also rich (9 out of 52) in small hydrophobic amino acids such as Ala and Val; (iv) it has a net positive charge; (v) it is essentially deficient (2 out of 52) in acidic amino acids. The cDNA for chromoplast CSase from *C. annuum* also contained transit peptide for targeting to plastids [13], although the cleavage site has not been determined in *C. annuum* CSase. The stretch of 5 amino acids (CK↓AVS) surrounding the cleavage site is highly con-

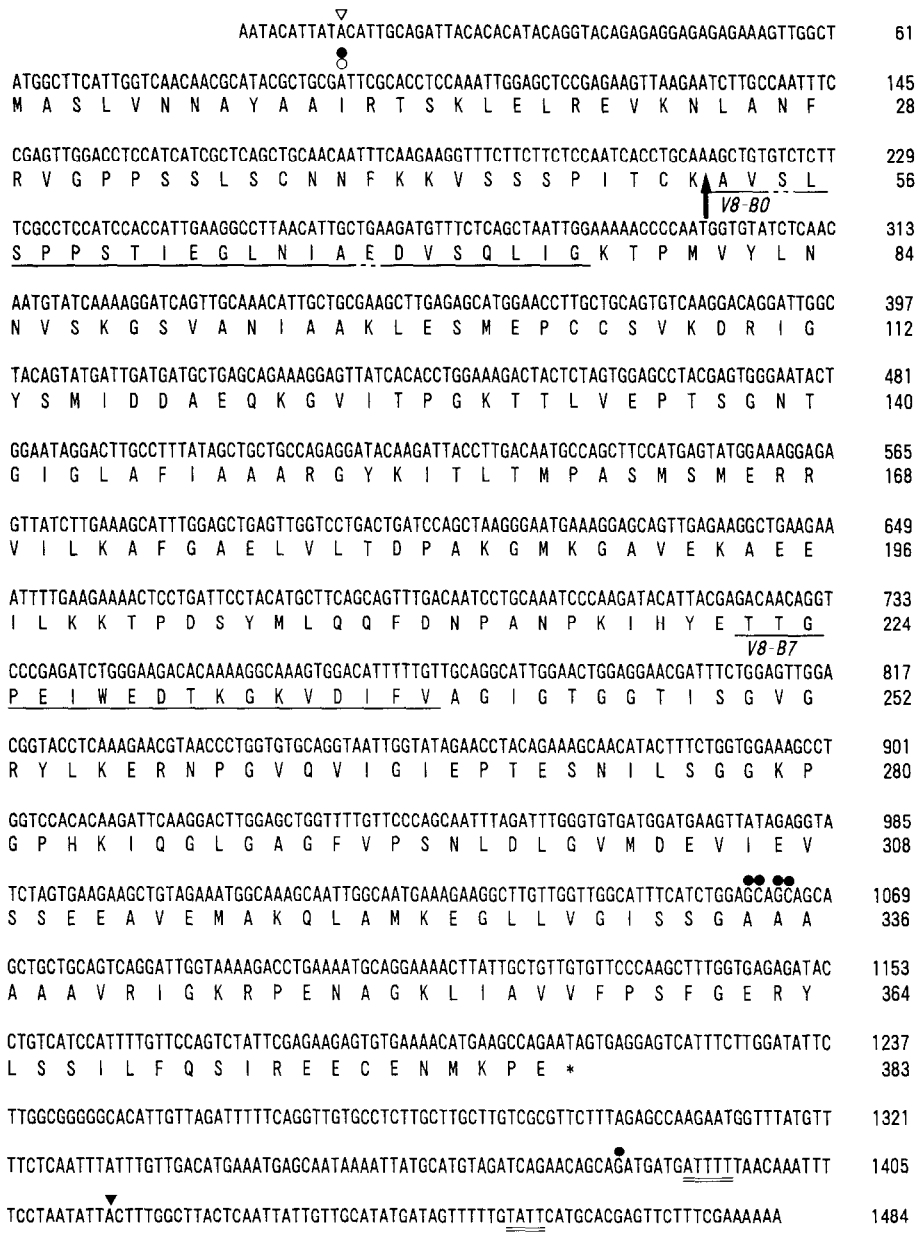

Fig. 1. Nucleotide and deduced amino acid sequence of a cDNA clone, λCSB11, encoding CSase B from spinach. The underlined amino acid sequences indicate the partial peptide fragments determined by protein microsequencing for the purified enzyme. Dotted underlining in fragment *V8-B0* indicates mismatching amino acids. Double underlining indicates putative polyadenylation signals. The arrow indicates the cleavage site of transit peptide. ▽, 5′ end of cDNA in λCSB2. ○, A was replaced with C in λCSB2. ▼, alternative poly (A) site of cDNA in λCSB2. ●, Mismatching with the sequence reported by Rolland et al. [12], A-95 → C, G-1061 → C, C-1062 → G, G-1064 → C, C-1065 → G, G-1384 → C.

served in both spinach and *C. annuum* proteins, suggesting the same proteolytic site in the *C. annuum* protein as in the spinach protein. The predicted secondary structures and the hydropathic features of two transit peptides are also similar, in particular, the region around the processing site (data not shown).

3.4. *Expression of spinach cysteine synthase B cDNA and genetic complementation in Cys*-E.coli

To confirm the identity of the isolated clones encoding CSase, a cysteine-auxotroph mutant, *E. coli* NK3, was genetically complemented by the expression of spinach CSase B. *E. coli* NK3 transformed with pCSB2, an expression vector containing full-length cDNA of cysteine synthase B under the *lacZ* promoter, was able to grow in the minimal medium without cysteine, similarly as pKM1 expressing spinach CSase A [6]; whereas *E. coli* transformed with a control vector, pUC19, was not (Fig. 3).

The expression of functionally active CSase B in *E.*

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                                    ↓
1. Consensus               1 : MAS..NN............E.......R.........N...KV......CKAVS.............I.......IG.TP.V.L............K.
2. CS B (Sp. oleracea)     1 : MASLVNNAYAAIRTSKLELREVKNLANFRVGPPSSLSCNNFKKVSSSPITCKAVSL--SPPSTIEGLNIAEDVSQLIGKTPMVYLNNVSKGSVANIAAKL
3. CS B (C. annuum)        1 : MASIINNPFTSL-CCNTNKCEPNRICSLRSQQSLVFD-NVNRKVGFPSVVCKAVSVQTKSPTEIEGLNIAEDVTQLIGNTPMVYLNTIVKGCVANIAAKL
4. CS A (T. aestivum)      1 :                                                                 MGEASSPAIAKDVTELIGNTPLVYLNKVTDGCVGRVAAKL
5. CS A (Sp. oleracea)     1 :                                                                  MVEEKAFIAKDVTELIGKTPLVYLNTVADGCVARVAAKL
6. cysK (E. coli)          1 :                                                                      MSKIFEDNSLTIGHTPLVRLNR--IGNGRIL-AKV
7. cysK (Sa. typhimurium)  1 :                                                                      MSKIYEDNSLTIGHTPLVRLN--RIGNGRIL-AKV
8. cysM (E. coli)          1 :                                                                         MSTLEQTIGNTPLVKLQRMGPDNGSEVWLKL

                         101 : E...P..SVK.RI...MI..AE..G...PG...L.E.T.GNTGI.LA..AA..GY.....MP..MS.ERR....A.GA.L.L.....GM.GA...A.E..
                         101 : ESMEPCCSVKDRIGYSMIDDAEQKGVITPGKTTLVEPTSGNTGIGLAFIAAARGYKITLTMPASMSMERRVILKAFGAELVLTDPAKGMKGAVEKAEEIL
                         101 : EIMEPCCSVKDRIGFSMISDAEEKGLISPGKTVLVEPTSGNTGIGLAFIAASRGYKLILTMPASMSLERRVILKAFGAELVLTDPAKGMKGAVSKAEEIL
                          41 : ESMEPCSSVKDRIGYSMITDAEEKGFIVPGKSVLIEPTSGNTGIGLAFMAAAKGYRLVLTMPASMSMERRIILKAFGAELILTDPLLGMKGAVQKAEELA
                          40 : EGMEPCSSVKDRIGFSMITDAEKSGLITPGESVLIEPTSGNTGIGLAFIAAAKGYKLIITMPASMSLERRTILRAFGAELILTDPAKGMKGAVQKAEEIR
                          36 : ESRNPSFSVKCRIGANMIWDAEKRGVLKPG-VELVEPTSGNTGIALAYVAAARGYKLTLTMPETMSIERRKLLKALGANLVLTEGAKGMKGAIQKAEEIV
                          36 : ESRNPSFSVKCRIGANMIWDAEKRGVLKPG-VELVEPTNGNTGIALAYVAAARGYKLTLTMPETMSIERRKLLKALGANLVLTEGAKGMKGAIQKAEEIV
                          32 : EGNNPAGSVKDRAALSMIVEAEKRGEIKPG-DVLIEATSGNTGIALAMIAALKGYRMKLLMPDNMSQERRAAMRAYGAELILVTKEQGMEGARDLALEMA

                         201 : .........L.QF.N..NP..H..TTGPEIW..T.G.........GT.GT..G.............................L...............
                         201 : KKTPDSY-MLQQFDNPANPKIHYETTGPEIWEDTKGKVDIFVAGIGTGGTISGVGRYL--KERNPGVQVIGIEPTES----NILSG--GKPGPHKIQGLG
                         201 : NNTPDAY-ILQQFDNPANPKIHYETTGPEIWEDTKGKIDILVAGIGTGGTISGTGRYL--KEKNPNIKIIGVEPTES----NVLSG--GKPG-------
                         141 : AKTPNSY-ILQQFENAANPKIHYETTGPEIWKGTGGKIDGLVSGIGTGGTITGTGKYL--QEQNPNIKLYGVEPTES----AILNG--GKPGPHKIQGIG
                         140 : DKTPNSY-ILQQFENPANPKVHYETTGPEIWKGTGGKIDIFVSGIGTGGTITGAGKYL--KEQNPDVKLIGLEPVES----AVLSG--GKPGPHKIQGLG
                         136 : ASNPEKYLLLQQFSNPANPEIHEKTTGPEIWEDTDGQVDVFIAGVGTGGTWTGVTPYIKGTKGKTDLISVAVEPTDSPVIAQALAGEEIKPGPHKIQGIG
                         136 : ASDPQKYLLLQQFSNPANPEIHEKTTGPEIWEDTDGQVDVFISGVGTGGTLTGVTRYIKGTKGKTDLITVAVEPTDSPVIAQALAGEEIKPGPHKIQGIG
                         132 : NRGEGK--LLDQFNNPDNPYAHYTTTGPEIWQQTGGRITHFVSSMGTTGTITGVSRFMR-------------EQSKPVTIVGLQPEEGSSIP-GIRRWP

                         301 : ....P........D................L...E....G.SSG.A...A.....................G.RYLS...F.............
                         301 : AGFVPSNLDLGVMDEVIEVSSEEAVEMAKQLAMKEGLLVGISSGAAAAAAVRIGKRPENAGKLIAVVFPSFGERYLSSILFQSIREECENMKPE
                         301 : --FIPGNLDQDVMDEVIEISSDEAVETAKQLALQEGLLVGISSGAAALAAIQVAKRPENAGKLIAVVFPSFGERYLSSILFQSIREECEKMKPEL
                         241 : AGFIPGVLDVDIIDETIQVSSDESIEMAKSLALKEGLLVGISSGAAAAAAIKVAQRPENAGKLFVVVFPSFGERYLSSVLFHSIKKEAESMVVE
                         240 : AGFIPGVLDVNIIDEVVQISSEESIEMAKLLALKEGLLVGISSGAAAAAAIKVAKRPENAGKLIVAVFPSFGERYLSSVLFDSVRKEAESMVIES
                         236 : AGFIPANLDLKLVDKVIGITNEEAISTARRLMEEEGILAGISSGAAVAAALKLQEDESFTNKNIVVILPSSGERYLSTALFADLFTEKELQQ
                         236 : AGFIPGNLDLKLIDKVVGITNEEAISTARRLMEEEVFLAGISSGAAVAAALKLQEDESFTNKNIVVILPSSGERYLSTALFADLFTEKELQQ
                         232 : TEYLPGIFNASLVDEVLDIHQRDAENTMRELAVREGIFCGVSSGGAVAGALRVAKANPDAV--VVAIICDRGDRYLSTGVFGEEHFSQGAGI
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Fig. 2. Comparison of deduced amino acid sequences of CSases from various sources and the consensus sequence The arrow indicates the cleavage site of the transit peptide. Dashes indicate gaps in sequence for the best alignment.

coli NK3 transformed with pCSB2 was also demonstrated by Western blot analysis and the enzymatic activity (Fig. 4). Molecular mass of the immuno-reactive protein derived from pCSB2 was calculated to be ca. 40 kDa. This suggested that the translation started from the proper ATG codon of pre-CSase B. The Shine–Dalgarno-like sequence (AGAAAG) is present 7 bp before the first ATG codon in the untranslated region of CSase B clone (Fig. 1). This sequence was presumably recognized as rRNA binding site in *E. coli*. The CSase enzymatic activity of translational product of pCSB2 was also demonstrated in the *E. coli* soluble protein. However, the activity of pCSB2 was lower than that of pKM1. This could be due either to the presence of transit peptide of no catalytic function or to the difference of construction of the chimeric genes; pKM1 was constructed with the coding region just 8 bp under the Shine–Dalgarno sequence of *lacZ* for efficient translation.

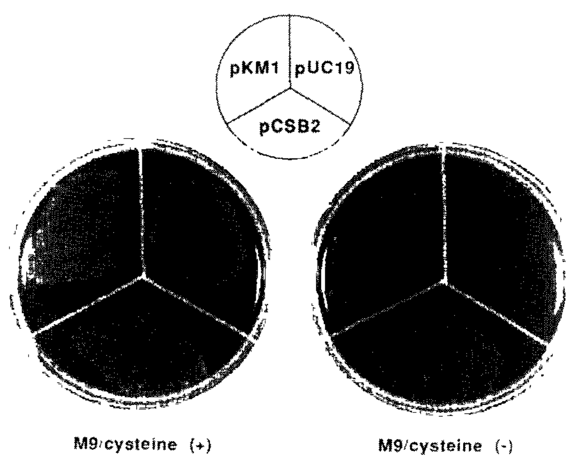

Fig. 3 Genetic complementation of Cys^- *E. coli* NK3 by transformation with expression vectors, pCSB2 and pKM1. Transformed bacteria were spread on M9 minimal agar plates supplemented with 0.02% leucine and tryptophan plus 0.5 mM cysteine (left plate) or without cysteine (right plate).

These expression studies in *E. coli* confirmed that this cDNA clone encodes CSase B that is functionally active in *E. coli* even with the transit peptide sequence.

3.5. *Hybridization analysis*

Southern blot analysis of genomic DNA indicated the presence of at least 2–3 copies *cysA* and *cysB* genes encoding CSase A and B, respectively, in spinach (Fig. 5). Presumably *cysA* and *cysB* comprise independent small multi-gene families and are expressed differently

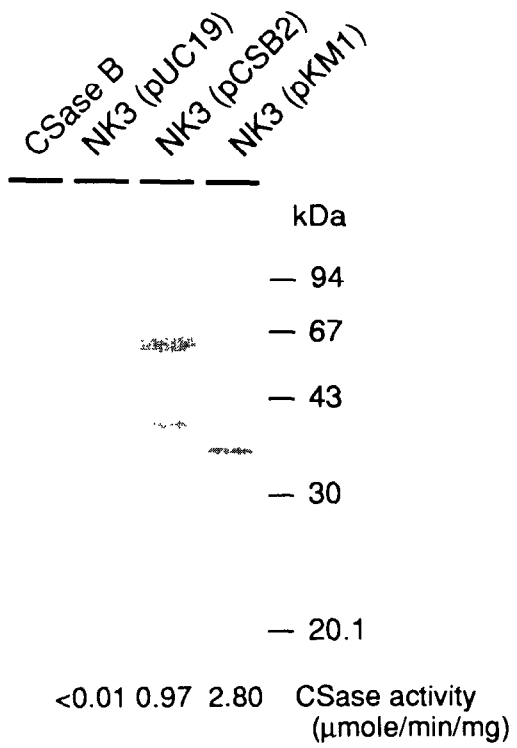

Fig. 4. Expression analysis of spinach CSase B in *E coli* NK3 by Western blotting and enzyme assaying For Western blotting, the protein was separated by 12% SDS-PAGE, transferred onto a Nylon filter, and then localized by immunostaining using rabbit anti-CSase A serum. For the enzyme assay, the activities of CSase were determined in cell-free extracts of *E. coli*. Lanes: CSaseB, purified spinach CSase B (0.25 μg); NK3 (pUC19), *E coli* protein 50 μg; NK3 (pCSB2), 50 μg; NK3 (pKM1), 5 μg.

during the developments as postulated in the case of glutamine synthase genes for ammonia fixation [19].

Expression of *cysB* gene encoding CSase B was analyzed in comparison with the *cysA* expression by RNA blot hybridization (Fig. 6). *CysB* gene was more expressed in leaves than roots. The small but substantial amounts of transcripts were also accumulated in roots, presumably due to the expression in non-green plastids. On the contrary, the expression of *cysA* gene was constitutive in leaves and roots of 4- and 10-week-old spinach.

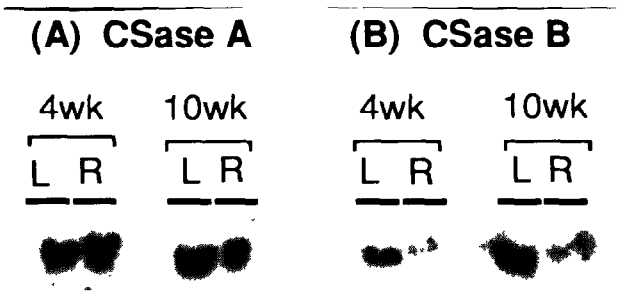

Fig. 6. Northern blot analysis of total spinach RNA Total RNA of leaves (L) and roots (R) of 4- and 10-week-old spinach was analyzed by probes for CSaseA and B. The transcript sizes hybridized with the probes were ca. 1.6 kb.

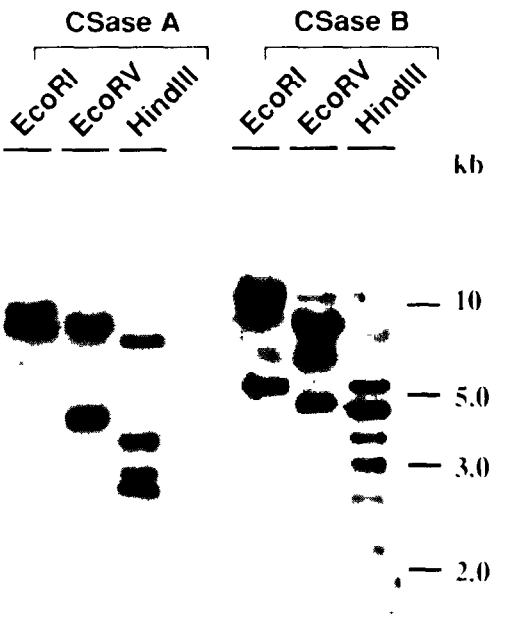

Fig. 5. Southern blot analysis of genomic DNA of spinach. The full-length cDNA clones of CSase A and B were used as probes. These probes cross-hybridized very weakly each other in the condition described in section 2.

These suggested that CSase B localized in chloroplasts is functionally related to sulfate reduction coupled with photosynthesis and utilizes sulfide immediately after the reduction of sulfate; whereas CSase A localized in cytoplasm has the general role for assimilation and detoxication of sulfide in the cells.

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