Characterization of an *Arabidopsis thaliana* cDNA encoding an S-adenosylmethionine-sensitive threonine synthase

Threonine synthase from higher plants

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Abstract An *Arabidopsis thaliana* cDNA encoding an S-adenosylmethionine-sensitive threonine synthase (EC 4.2.99.2) has been isolated by functional complementation of an *Escherichia coli* mutant devoid of threonine synthase activity. Threonine synthase from *A. thaliana* was shown to be synthesized with a transit peptide. The recombinant protein is activated by S-adenosylmethionine in the same range as the plant threonine synthase and evidence is presented for an involvement of the N-terminal part of the mature enzyme in the sensitivity to S-adenosylmethionine.

Key words: Threonine synthase; *o*-Phospho-L-homoserine; S-adenosylmethionine; Pyridoxal 5'-phosphate; Chloroplast; *Arabidopsis thaliana*

1. Introduction

In plants and microorganisms threonine synthesis is part of a multibranched biosynthetic pathway originating with aspartate and leading to the synthesis of lysine, methionine, threonine and isoleucine. Threonine synthase (EC 4.2.99.2), a pyridoxal 5'-phosphate (PLP)-dependent enzyme, catalyses the last step of threonine formation, converting *o*-phospho-L-homoserine (OPH) to threonine and inorganic phosphate [1–3]. In plants, OPH is also involved in methionine synthesis [4] whereas in bacteria and fungi, homoserine is the branch point intermediate between threonine and methionine biosynthetic pathways. Therefore, the regulatory patterns of threonine synthase in plants may be expected to be different from those in bacteria and fungi.

Threonine synthase has been purified from *Neurospora crassa* [5] and from *Escherichia coli* [6]. The corresponding gene has been isolated from a number of bacteria [6–11] and from *Saccharomyces cerevisiae* [12]. In plants, only partial purification of threonine synthase has been achieved [13–15]. The enzyme was shown to be exclusively confined to the chloroplast [16]. Of main interest is a marked sensitivity of plant threonine synthase activity to S-adenosylmethionine (SAM), a methionine derivative [13–15]. The interaction between SAM and plant threonine synthase is cooperative and reversible, resulting in 20-fold enzyme activation. These characteristics, not observed with the bacterial and fungal enzymes, are indicative of a functional regulatory enzyme [17]. We report here the isolation of a cDNA clone encoding a SAM-sensitive threonine synthase from plants.

2. Materials and methods

2.1. Material

The *E. coli* threonine auxotroph GIF 41 (*thrC 1001 thi-1 relA spoT1*) was a kind gift of Dr. I. Saint-Girons (Institut Pasteur, Paris). This strain was maintained on minimal medium M9 [18] supplemented with 0.2% glucose (w/v), 0.3 mM L-threonine and 30 μM thiamine. The λYES-R cDNA expression library was prepared with mRNAs from the above-ground parts of *A. thaliana* plants at all stages of development and converted into a plasmid library (pYES), carrying carbenicillin resistance [19]. *o*-Phospho-L-homoserine was synthesized essentially as described by Rognes [20] according to the modifications of Ravanel [21]. *A. thaliana* (var. *columbia*) plants were grown in soil at 25°C with a 12-h photoperiod.

2.2. Functional complementation and determination of enzymatic activity

E. coli GIF 41 mutant competent cells were transformed by electroporation with the pYES library [22]. Then, cells were washed twice in M9 medium and plated on M9 medium supplemented with 30 μM thiamine, 100 μg/ml carbenicillin, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 0.2% glucose (w/v). Up to 12 10^6 transformants were screened. Plasmids were isolated [18] from colonies growing on the selection medium, and the original GIF 41 strain was transformed again with the purified plasmids to confirm the functional complementation.

Soluble protein extracts from overnight cultures of the complemented clones induced with 1 mM IPTG were prepared in buffer A (50 mM Na-HEPES, pH 7.5, 1 mM Na_2-EDTA, 1 mM DTT) containing protease inhibitors (5 mM ε-aminocaproic acid and 1 mM benzamidine). Proteins were measured by the method of Bradford [23] using bovine γ-globulin as a standard.

Threonine synthase activity was measured in a volume of 100 μl containing 100 mM Na-HEPES, pH 8.0, 1 mM OPH, 10 mM NaF, 50 μM PLP, in the absence or presence of 200 μM SAM. Assays were initiated by adding enzyme. After incubation at 30°C for 5–60 min, reactions were stopped by addition of 50 μl of 20% (w/v) TCA and the precipitated proteins removed by centrifugation. L-Threonine formation was determined by high performance liquid chromatography (HPLC) after derivatization of 2–20 μl of the TCA supernatants with *o*-phthaldialdehyde (OPA) [24]. The samples (10–50 μl) were injected onto a Novapack C18 (3.9×150 mm, 4 μm particle size, Millipore waters) column connected to a HPLC system. OPA derivatives were eluted in isocratic conditions (67.2 mM sodium acetate, pH 4.5; 16.8% (v/v) acetonitrile; flow rate, 1 $ml \cdot min^{-1}$) and detected by fluorescence measurement at 455 nm after excitation at 340 nm using a SFM 25 fluorimeter (Kontron). Quantitative analysis of amino acids

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Abbreviations: DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; OPA, *o*-phthaldialdehyde; OPH, *o*-phospho-L-homoserine; PLP, pyridoxal 5'-phosphate; SAM, S-adenosylmethionine

The nucleotide sequence reported in this paper has been deposited in the EMBL database with accession number L41666.

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was carried out by measuring peak areas using a chromatography data system 450-MT2 software (Kontron) and solutions of amino acids of known concentrations as standard.

2.3. cDNA sequencing and PCR amplification of 5′ sequence

The cDNA inserts were subcloned into the *Xho*I site of pBluescript II SK(−) (Stratagene). DNA sequence analysis was carried out on both strands using the dideoxynucleotide chain termination method [25]. PCR amplification [26] of the 5′ end of threonine synthase cDNA was carried out on a λgt11 cDNA expression library (Clontech). A reverse 25-mer oligonucleotide specific for the 5′ end of pYES TS 3 cDNA (5′-GGCCATGTGCTTTTACCAACACGCG-3′) was used in conjunction with the λgt11 forward primer. PCR products resulting from independent PCR reactions were sequenced on both strands by automatic fluorescence-based sequencing of templates amplified by PCR (Génome Express).

2.4. Purification of recombinant threonine synthase (TS 1)

A soluble protein extract (4.5 g) from an overnight culture of *E. coli* GIF 41 (pYES TS 1) induced with 1 mM IPTG was prepared in 250 ml of buffer A containing protease inhibitors and applied onto a DEAE-Trisacryl M^+ column (5.5×20) previously equilibrated with buffer A. Proteins were eluted with a linear gradient of NaCl (0–250 mM in 900 ml of buffer A). Threonine synthase activity was eluted as a single peak at a concentration of 150 mM NaCl. The fractions containing threonine synthase activity (540 mg) were concentrated, desalted on Mini-ultrasette (Filtron) and then applied onto a Red Sepharose CL 6 B column (2.5×16 cm) previously equilibrated in buffer A. Proteins were eluted with a linear gradient of NaCl (0–300 mM in 270 ml of buffer A). Threonine synthase activity was eluted between 150 and 180 mM NaCl. Fractions containing threonine synthase activity (23 mg) were concentrated, desalted on Macrosep 30 K (Filtron) and applied onto a MonoQ HR 10/10 column equilibrated in buffer A. Proteins were eluted with a linear gradient of NaCl (0–180 mM, in 80 ml of buffer A) followed by a 30 ml step in buffer A containing 180 mM NaCl. Threonine synthase activity was eluted at 180 mM NaCl. The most active fractions (2.8 mg) were concentrated and injected on a Superdex 200 column (1.6×60 cm) equilibrated in buffer A containing 50 mM NaCl. Elution was performed with the equilibrating buffer at a flow rate of 1 ml·min^{-1}. Threonine synthase was eluted with 75 ml of buffer. The active fractions containing pure threonine synthase (2 mg) were desalted and concentrated on Macrosep 30 K. Purified recombinant threonine synthase was used to raise specific antibodies in a rabbit and IgGs were purified from the antisera [27].

2.5. Purification of threonine synthase from A. thaliana *leaves and N-terminal sequencing*

A. thaliana leaves (500 g) were homogenized in buffer A containing protease inhibitors and the supernatant solution obtained by centrifugation was fractionated with $(NH_4)_2SO_4$. The 40–65% precipitate was collected by centrifugation at 30 000×*g* for 30 min, dissolved in 60 ml of buffer A containing protease inhibitors and dialysed overnight against the same buffer. The protein extract (5.2 g, 70 ml) was fractionated using successively a DEAE-Trisacryl M^+ column and a Red Sepharose CL 6 B column in the same conditions as for TS 1 purification. The fraction containing partially purified threonine synthase (1.4 mg) was subjected to preparative SDS-PAGE [28] and threonine synthase polypeptide (as identified by Western blotting [29] using the specific antibodies) was extracted from the corresponding gel slice by electroelution in a buffer containing 50 mM NH_4CO_3 and 0.1% SDS (v/v). N-terminal amino acid sequencing was carried out with a Model 477 A gas–liquid-phase protein sequencer (Applied Biosystems).

3. Results

3.1. Functional complementation of the thrC mutation in E. coli

The *E. coli* threonine auxotroph GIF 41 was transformed with plasmid DNA from an amplified *A. thaliana* pYES cDNA expression library that initially contained 10^7 independent recombinants [19]. Of approximately 10^6 transformants, three different clones, designated as pYES TS 1–3 were obtained on selection for carbenicillin resistance and threonine prototrophy. Restriction patterns and partial sequence analyses indicated that the cDNAs isolated were identical in sequence yet truncated on their 5′ end. To provide unequivocal evidence that these cDNAs encoded threonine synthase, we first measured this enzyme activity in crude protein extracts prepared from pYES TS 1–3 and from *E. coli* GIF 41 mutant, wild-type *E. coli* DH10B strain and *A. thaliana* leaves. Threonine synthase activity was measured in absence or presence of SAM, the activator of the plant enzyme [15]. As shown in Table 1, threonine synthase activity was not detected in the original mutant strain and no stimulation of wild-type *E. coli* enzyme by SAM was observed. On the contrary, expression of the cDNA inserts correlated with the presence of a SAM-sensitive threonine synthase activity in the soluble protein extract from the complemented bacteria. Surprisingly, the extent of SAM stimulation depends on the length of the N-terminal coding sequence of the cDNA. Thus, as indicated in Table 1, recombinant threonine synthase encoded by the longest cDNA (pYES TS 3) is stimulated by SAM in the same range (around 20-fold) as threonine synthase from a crude extract of *A. thaliana* leaves. On the contrary, threonine synthase encoded by p YES TS 2 and by the shortest cDNA insert (pYES TS 1) are stimulated by SAM only 10- and 3-fold, respectively.

Table 1
Threonine synthase activity measurements in *E. coli* GIF 41 complemented clones, in control strains and in *A. thaliana* crude extract

Source	Length of the cDNA coding sequence (bp)	Specific activity[a] without SAM	Specific activity with SAM (200 μM)	Fold stimulation
DH10B	–	0.98	0.98	1
GIF 41	–	n.d.[b]	n.d.	–
GIF 41 (pYES TS 1)	1362	24.6	74.9	3
GIF 41 (pYES TS 2)	1410	1.4	13.8	10.4
GIF 41 (pYES TS 3)	1458	2	42.9	21.7
A. thaliana crude extract	–	0.036	0.72	20

Cultures of *E. coli* strains were induced overnight with 1 mM IPTG and protein extracts were prepared. Threonine synthase activity was measured with or without SAM (200 μM) in soluble protein extracts from wild-type *E. coli* strain and from complemented and non-complemented *E. coli* (GIF 41) strains. These activities are compared with threonine synthase activity of a soluble protein extract prepared from *A. thaliana* leaves. These data are from a representative experiment. High activity of TS 1 recombinant threonine synthase without SAM was explained by a 10-fold higher level of expression compared with TS 2 and TS 3 as estimated by dot-blot experiments using polyclonal anti-TS1 antibodies and soluble protein extracts prepared from pYES TS 1–3.

[a]Specific activity is expressed as nmol theronine formed·min^{-1}·mg^{-1} protein.

[b]Not detectable.

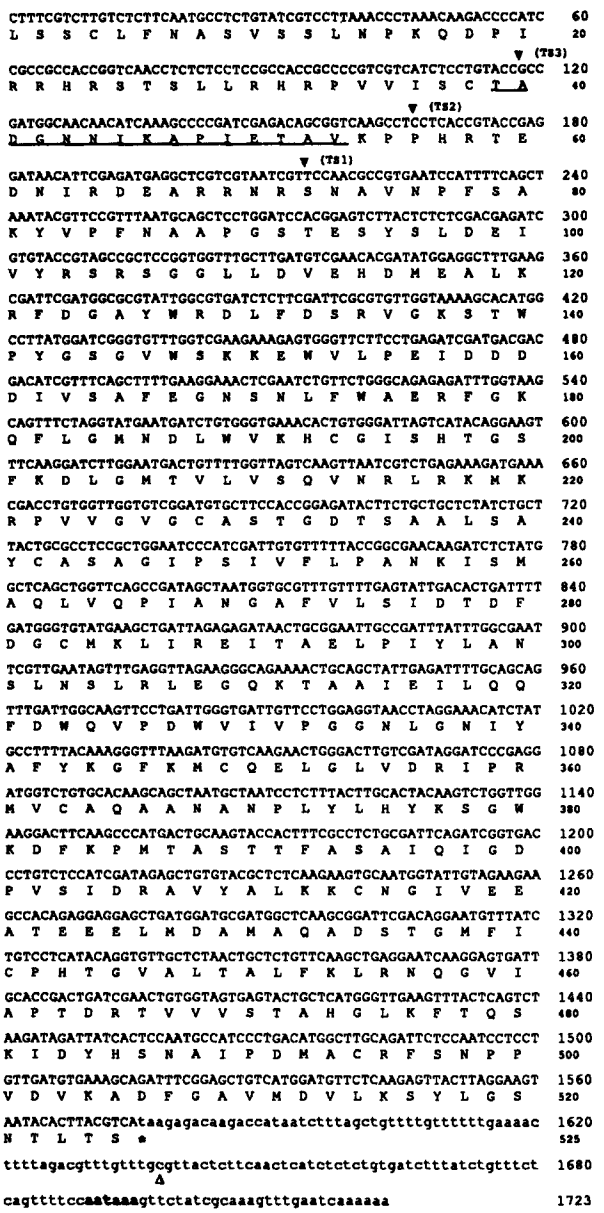

Fig. 1. Nucleotide and predicted amino acid sequences of the cDNA encoding *A. thaliana* threonine synthase. The nucleic acid sequence is presented on the top line with the derived amino acid sequence below. Nucleotides and amino acids are numbered on the right of the sequence. The beginning of pYES TS 1–3 cDNA is indicated (▼). The stop codon is marked with an asterisk and the 3′ untranslated region is shown in lower case type. The putative polyadenylation signal is indicated in bold. The triangle (△) shows the alternative position of the poly(A) tail in pYES TS 2 cDNA. The 117 first bases were determined using PCR amplification. The N-terminal sequence derived from Edman degradation of the mature protein purified from *A. thaliana* leaves is underlined.

3.2. Characterization of threonine synthase cDNA and deduced amino acid sequence analysis

Sequence analysis reveals that the open reading frame of the longest cDNA isolated codes for a protein devoid of initiating methionine, indicating that the sequence is not full length. Up to 12 10^6 additional transformants were screened for functional complementation but among the 18 cDNAs isolated none was full length. To find the lacking 5′ side of the cDNA, PCR amplification was realized using λYES, λZAP and λgt 11 *A. thaliana* cDNA libraries. The largest fragment (240 bp), isolated by PCR amplification of a λgt 11 library, overlapped the TS 3 cDNA 5′ sequence over 123 nucleotides and extended upstream on 117 additional nucleotides (39 amino acids). Unfortunately, this sequence appeared to be again truncated in its 5′ part since no initiating methionine was found. In spite of numerous attempts, no longer cDNA sequence could be determined by this method and no expressed sequence tag (EST) corresponding to the 5′ end of our clone was found in databases. The nucleotide sequence obtained (Fig. 1) contains an open reading frame of 1575 bp encoding a predicted protein of 525 amino acids with a calculated molecular mass of 57 662 Da. Threonine synthase was isolated from *A. thaliana* plants as indicated in Section 2. Its N-terminal sequence was determined and gave a sequence of 15 residues (T-A-D-G-N-N-I-K-A-P-I-E-T-A-V). This sequence corresponds to the amino acid sequence (position 39–53) deduced from the cDNA (Fig. 1). Therefore, plant threonine synthase is synthesized with a N-terminal presequence of at least 38 residues that is absent in the mature form.

The 3′ untranslated region is 64 or 139 nucleotides long depending on the cDNA clones. For the shortest untranslated region (pYES TS 2 cDNA) no consensus polyadenylation signal was found. On the contrary, a typical eukaryotic consensus polyadenylation signal sequence (AATAAA) [30] is located 22 bp upstream from the poly(A) tail in the largest cDNA clone.

3.3. Amino acid sequence comparison

A search of the GenBank data base revealed significant homologies between *Bacillus subtilis thrC* gene product (accession number X04603) (24% identity, 33% similarity) and the deduced amino acid sequence of the threonine synthase cDNA isolated. The similarity of *A. thaliana* enzyme with other threonine synthases (*C. glutamicum* (accession number X56037), *E. coli* (accession number J01707), *P. aeruginosa* (accession number X65033), *S. cerevisiae* (accession number X17256)) was much lower (< 16%). When all the sequences are aligned (Fig. 2), six conserved blocks containing identical and similar amino acids are found. The comparison also shows that mature *A. thaliana* threonine synthase presents an amino-terminal extension of 51 and 103 amino acids compared with *E. coli* and *B. subtilis* threonine synthases, respectively.

3.4. A. thaliana *leaves contain a single isoform of threonine synthase*

Soluble protein extracts from *A. thaliana* leaves and from *E. coli* GIF 41 (pYES TS 3) were fractionated in the same conditions on a MonoQ HR 5/5 column (Fig. 3A). Plant threonine synthase and recombinant threonine synthase activities were eluted in a single peak of activity at the same ionic strength (150 mM NaCl) indicating that a single form of threonine synthase was present in *A. thaliana* leaves. SDS-PAGE and Western blot analyses conducted with pooled active fractions from both chromatographies (Fig. 3B) show that the anti-TS 1 antibodies react with a single polypeptide of M_r 55 000 ± 1000 in both the *E. coli* pYES TS 3 and *A. thaliana* leaf extract. Together, these data suggest that the protein encoded by pYES TS 3 corresponds to the unique form of threonine synthase detected in *A. thaliana* leaves.

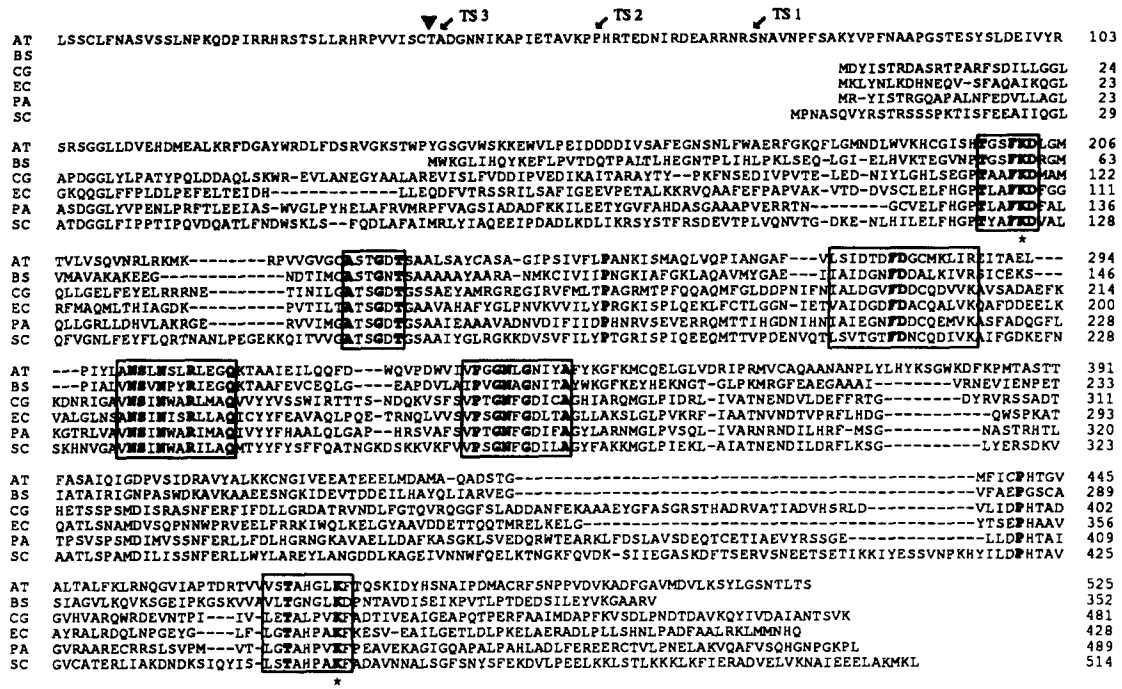

Fig. 2. Comparison of derived amino acid sequences of threonine synthase from *A. thaliana* with that from *Bacillus subtilis* (BS) [7], *Corynebacterium glutamicum* (CG) [8], *E. coli* (EC) [6], *Pseudomonas aeruginosa* (PA) [11] and *Saccharomyces cerevisiae* (SC) [12]. Dashes indicate gaps introduced to optimize the alignment. Identical amino acids are in boldface. Conserved sites containing identical and similar residues are boxed (accepted alternatives are I-L-V-M, D-E, R-K, S-T, F-Y). The asterisks indicate the conserved lysine residues which could be involved in pyridoxal 5′-phosphate binding. The beginning of the mature *A. thaliana* threonine synthase (▼) and of the different clones (↙) is indicated.

3.5. Molecular mass of native threonine synthase

Gel filtration chromatography on Superdex 200 was conducted with partially purified threonine synthase from *A. thaliana* leaves and with a crude extract from *E. coli* GIF 41 (pYES TS 3). In both cases, a native molecular mass of approximately 110 kDa was determined from threonine synthase activity measurements. Therefore the native plant and recombinant proteins appear to be homodimers. The shortest recombinant protein (TS 1) also appeared to be a homodimer as determined by gel filtration in the last step of its purification.

4. Discussion

This is the first report on the isolation and characterization of a cDNA corresponding to the mRNA for plant threonine synthase. Confidence in the identity of the cDNA comes from the following considerations. Firstly, expression of pYES TS 3 complements the *thrC* mutation of the *E. coli* GIF 41 strain. Expression of this cDNA correlated with a detection of a threonine synthase activity in a soluble protein extract from the complemented bacteria. Secondly, whereas the activity of bacterial threonine synthases is not affected by SAM, the recombinant threonine synthase is strongly stimulated by this methionine derivative as is the enzyme activity from an *A. thaliana* crude extract. Thirdly, antibodies raised against recombinant threonine synthase recognize a single polypeptide corresponding to threonine synthase in *A. thaliana* leaves. Finally, the N-terminal sequence derived from Edman degradation of the mature protein purified from *A. thaliana* leaves matches the sequence deduced from the cDNA (position 39–53), confirming the identity between the plant and cloned enzyme. Moreover, the first 38 amino acids deduced from the cDNA sequence appeared to be absent in threonine synthase purified from leaves and could therefore correspond to the end of a chloroplastic transit peptide, in agreement with the demonstration that threonine synthase is confined to the chloroplast in pea and barley leaves [16].

Comparison of threonine synthase amino acid sequences indicates that *A. thaliana*, yeast and bacterial threonine synthase peptide sequences present six sites of strictly conserved amino acids (Fig. 2). These highly conserved regions may include amino acids required for enzyme activity, that is the site for OPH recognition and that for PLP binding. The participation of PLP in the reaction implies the formation of a Schiff base with the ε-NH_2 moiety of a lysine residue in the protein. This lysine residue has never been biochemically determined for any threonine synthase. As Fig. 2 shows, there are two lysine residues (indicated by asterisks) that are conserved between the six sequences. Amino acid comparisons [7,31] between evolutionarily related PLP-dependent enzymes suggest that Lys-107 in *E. coli* threonine synthase is the PLP binding site. Consequently, Lys-202 in *A. thaliana* threonine synthase which is aligned with Lys-107 in *E. coli* threonine synthase may be linked to PLP. Remarkably, mature plant threonine synthase is characterized by a N-terminal extension of 51 and 103 amino acids compared with *E. coli* and *B. subtilis* enzymes, respectively. This N-terminal extension of the plant enzyme could be of functional importance as indicated by the enzyme activity measurements reported in Table 1. Indeed, TS 1 protein which is truncated by 33 amino acids on its N-terminal part compared to the mature form of the enzyme remains active but appeared to be poorly stimulated by SAM (3-fold stimulation versus 20-fold stimulation for the mature form). This dramatic reduction of the stimulation by SAM observed for TS 1 suggests a role of the N-terminal part

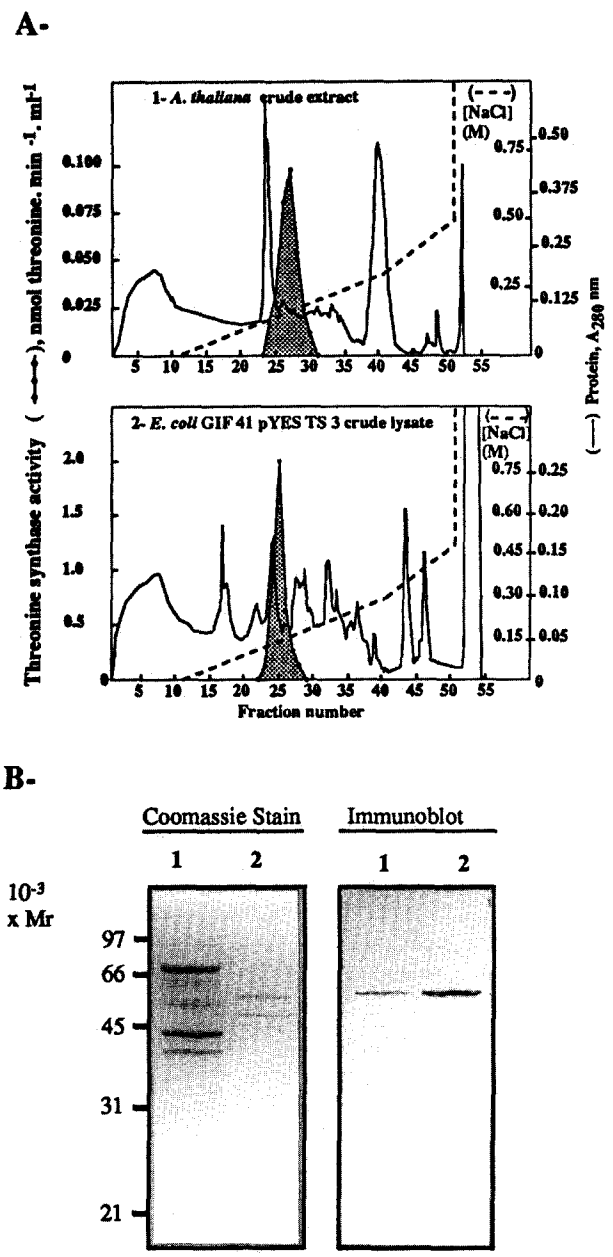

Fig. 3. Fractionation of soluble protein extracts from *A. thaliana* leaves and from *E. coli* (pYES TS 3) on MonoQ HR 5/5 column and Western blot analysis of the threonine synthase active fractions. (A) Protein extracts (10 mg protein) were loaded on an anion-exchange (MonoQ HR5/5) column and eluted with a linear NaCl (0–300 mM) gradient in buffer A containing a mixture of protease inhibitors. The sample loaded were total soluble protein extract from (1) *A. thaliana* leaves and (2) *E. coli* GIF 41 (pYES TS 3). Threonine synthase activity is expressed as nanomole threonine formed·min^{-1}·ml^{-1} in the presence of 200 μM SAM. (B) Samples from threonine synthase active fractions were separated on SDS-PAGE gels (12% acrylamide (w/v)) and stained with Coomassie brilliant blue R-250 or electrotransfered onto nitrocellulose and immunostained using purified IgGs raised against recombinant threonine synthase (TS 1) at a dilution of 1 : 10 000. Lane 1, *A. thaliana* active fraction, 12 μg; lane 2, TS 3 active fraction, 1.2 μg.

of plant threonine synthase in enzyme stimulation by SAM. This difference in sensitivity is not attributable to a difference in oligomerization state of the enzyme since both TS 1 and TS 3 appeared to be homodimers as determined by gel filtration analyses. Biochemical characterization of the mature enzyme and of truncated forms are in progress to confirm this observation.

Our results indicate that a single peak of activity is detectable in a fractionated total soluble protein extract from *A. thaliana* leaves and the identity between the cloned enzyme and this activity is demonstrated. These results are consistent with previous reports [15,16] describing a single chloroplastic threonine synthase in plant leaves. However, in the progress of our work, a cDNA sequence (ATTHRC) encoding an *A. thaliana* threonine synthase was deposited in the data bank GenBank (accession number Z46263). This cDNA sequence has been isolated by functional complementation in yeast (S.F. Aas and S.E. Rognes, 1994, unpublished). Interestingly, the deduced amino acid sequence of this cDNA is only 11% identical with TS 3. On the contrary, ATTHRC threonine synthase is very similar to *S. cerivisiae* threonine synthase (56% identity and 66% similarity). The isolation of this second sequence raises the question of the existence of two threonine synthase isoforms in *A. thaliana*. Although a single threonine synthase activity is detectable in our conditions in *A. thaliana* leaves as well as in other plants [20], another enzyme could be expressed in plants at a very low level that was not detectable with our present methodology to assess enzyme activity. Alternatively, this putative plant threonine synthase could be expressed in conditions not recovered when the cDNA library was established. Further work is needed to characterize the biochemical properties and subcellular localization of this putative second threonine synthase and to integrate these results with the proposed regulatory patterns [17].

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