The final step of pantothenate biosynthesis in higher plants: cloning and characterization of pantothenate synthetase from *Lotus japonicus* and *Oryza sativum* (rice)

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We have isolated a *Lotus japonicus* cDNA for pantothenate (vitamin B_5) synthetase (PS) by functional complementation of an *Escherichia coli panC* mutant (AT1371). A rice (*Oryza sativum*) expressed sequence tag, identified by sequence similarity to PS, was also able to complement the *E. coli* auxotroph, as was an open reading frame from *Saccharomyces cerevisiae* (baker's yeast). The *Lotus* and rice cDNAs encode proteins of approx. 34 kDa, which are 65% similar at the amino acid level and do not appear to encode *N*-terminal extensions by comparison with PS sequences from other organisms. Furthermore, analysis of genomic sequence flanking the coding sequence for PS in *Lotus* suggests the original cDNA is full-length. The *Lotus* and rice PSs are therefore likely to be cytosolic. Southern analysis of *Lotus* genomic DNA indicates that there is a single gene for PS. Recombinant PS from *Lotus*, overexpressed in *E. coli* AT1371, is a dimer. The enzyme requires D-pantoate, β-alanine and ATP for activity and has a higher affinity for pantoate (K_m 45 μM) than for β-alanine (K_m 990 μM). Uncompetitive substrate inhibition becomes significant at pantoate concentrations above 1 mM. The enzyme displays optimal activity at about 0.5 mM pantoate (k_{cat} 0.63 s^{-1}) and at pH 7.8. Neither oxopantoate nor pantoyllactone can replace pantoate as substrate. Antibodies raised against recombinant PS detected a band of 34 kDa in Western blots of *Lotus* proteins from both roots and leaves. The implications of these findings for pantothenate biosynthesis in plants are discussed.

Key words: functional complementation, overexpression in *Escherichia coli*, substrate inhibition, vitamin B_5, Western-blot analysis.

INTRODUCTION

Pantothenic acid (vitamin B_5) is the common essential precursor to CoA and acyl carrier protein, which are cofactors required in many energy-yielding reactions and also by a large number of metabolic enzymes [1]. CoA is also involved in the regulation of key metabolic reactions such as those catalysed by pyruvate dehydrogenase [2]. Biosynthesis of pantothenate takes place in bacteria, fungi and plants only, while animals must obtain it from their diet [3]. The pathway is best understood in *Escherichia coli*, where it comprises four enzymic steps [4–7]. Pantothenate is synthesized by the condensation of pantoate, derived from α-oxoisovalerate, the oxo acid of L-valine, and β-alanine, produced by the decarboxylation of L-aspartate (see Figure 1 in [6]). The ATP-dependent condensation of pantoate and β-alanine is catalysed by pantothenate synthetase (PS; EC 6.3.2.1), which is encoded by the gene *panC*. This condensation is thought to proceed via a pantoyl adenylate intermediate which reacts with β-alanine to give pantothenate, AMP and pyrophosphate [8,9].

Pantothenate may be synthesized by a similar route in plants. First evidence for this came from a pantothenate auxotroph of *Datura innoxia* (thorn-apple) [10]. Pantothenate, oxopantoate and pantoate (intermediates in the microbial pathway), supported the growth of the mutant in cell culture, whereas α-oxoisovalerate did not [11]. This implies that oxopantoate and pantoate are precursors for pantothenate in *Datura* also. The apparent inability of the mutant to produce oxopantoate may be interpreted in terms of a deficiency in oxopantoate hydroxymethyltransferase (OPHMT), the first enzyme on the *E. coli* pathway (for a review, see [12]). Attempts to detect OPHMT activity in wild-type *Datura* did not meet with success [11]. Similarly, enzyme analysis of pantothenate biosynthesis in various plant species and tissues was carried out in our laboratory with inconclusive results (C. E. Jones and U. Genschel, unpublished work). However, this may well reflect a low rate of flux through the pantothenate pathway in plants, rather than any substantive differences. Another indication that the plant biosynthetic pathway for pantothenate is similar to that in bacteria came from a feeding study with pea (*Pisum sativum*) leaf discs [13]. This demonstrated the incorporation of radiolabel from [^{14}C]valine into α-oxoisovalerate, oxopantoate and pantoate.

Given the difficulties with biochemical analysis of the pantothenate biosynthetic pathway in plants, a genetic strategy seemed more promising in order to establish the pathway beyond doubt. Here we report the isolation and characterization of *Lotus japonicus* (a plant related to bird's-foot trefoil, *L. corniculatus*)

Abbreviations used: EST, expressed sequence tag; OPHMT, oxopantoate hydroxymethyltransferase (= ketopantoate hydroxymethyltrasnferase, 'KPHMT'); ORF, open reading frame; PS, pantothenate synthetase; GCG, (University of Wisconsin) Genetics Computer Group; iPCR, inverse PCR.

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The nucleotide sequence data reported here have been submitted to the EMBL, GenBank® and DDBJ Nucleotide Sequence Databases under the accession numbers Y10252 (*Lotus japonicus panC*) and Y10253 [rice (*Oryza sativum*) *panC*].

and rice cDNAs for PS and verification of a yeast open reading frame (ORF) as that for PS. This is the first report of eukaryotic genes that participate in pantothenate biosynthesis and represents the first concrete evidence for the pathway in plants. Purification of recombinant *Lotus* PS has enabled functional characterization of the enzyme and its detection in plant tissue.

MATERIALS AND METHODS

Materials

All reagents were of the highest available grade and, unless otherwise indicated, were purchased from the Sigma Chemical Co., Poole, Dorset, U.K. Oxopantoyl-lactone was prepared from DL-pantoyl-lactone according to the method of Ojima et al. [14]. Potassium oxopantoate was prepared by dissolving oxopantoyl-lactone in water and adjusting the pH to 7.00 with KOH. Potassium D-pantoate was prepared by incubating D-pantoyl-lactone with an equimolar amount of KOH for at least 2 h at room temperature, and then adjusting the pH to between 7.00 and 8.00. *Lotus japonicus* (Regel) Larsen (GIFU B-129-S9) was grown from seeds in a mixture of vermiculite and potting compost at 20 °C in a growth chamber.

General methods

The molecular-biology protocols, unless otherwise stated, were as described by Sambrook et al. [15]. Oligonucleotide synthesis, DNA sequencing and amino acid sequencing were carried out as a service provided by the Protein and Nucleic Acid Chemistry Facility (PNACF), Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge, Cambridge, U.K. Computer analysis of the DNA and protein sequences were carried out using the Wisconsin Package Version 9.0 {Genetics Computer Group (GCG), Madison, WI, U.S.A. [16]}. Nucleotide databases were searched using the BLAST algorithm [17] network service at the National Center for Biotechnology Information (NCBI), Bethesda, MD, U.S.A. Genomic DNA from *Lotus japonicus* was prepared as described by Dellaporta et al. [18] from leaves of about-8-week-old plants, and genomic Southern analysis was carried out essentially as described by [19].

E. coli DH5α was the general host for subcloning and plasmid propagation. Functional complementation experiments and subsequent over-expression of recombinant PS were carried out in the *E. coli panC* mutant AT1371 {*panC4,* Δ(gpt-proA)62, lacY1, tsx-29, glnV44(AS), galK2, LAM-, rac-0, hisG4(Oc), rfbD1, xylA5, mtl-1, argE3(Oc), thi-1; [5]} which was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT, U.S.A. Plasmid DNA from *E. coli* strains was purified using the QIA spin-prep columns kit (Qiagen). All bacterial cultures were grown in culture flasks and incubated at 37 °C in a rotary incubator shaking at 180 rev./min.

Protein concentrations were determined by the method of Bradford [20] using the Bio-Rad protein assay kit with BSA as standard. Routine SDS/PAGE was carried out using the method of Laemmli [21] and proteins were stained with an equal mixture of Coomassie Blue R250/G250 stain.

Isolation of a *Lotus japonicus* cDNA for PS

A *Lotus* cDNA library in pBluescript was generated by mass excision of the plasmid from a *Lotus japonicus* root nodule cDNA library in λZAP II (a gift from Dr. J. Stougaard, University of Aarhus, Aarhus, Denmark) using the EXASSIST/SOLR system (Stratagene). Competent *E. coli* AT1371 cells ($CaCl_2$) were transformed with the plasmid cDNA library and washed with unsupplemented GB1 minimal medium (comprising 100 mM potassium phosphate, 2 g/l $(NH_4)_2SO_4$, 4 g/l glucose, 0.25 g/l $MgSO_4$,$10H_2O$, 0.25 mg/l $FeSO_4$,$7H_2O$ and 5 mg/l thiamine, pH 7.0) prior to plating on solidified GB1 medium (1.5% Oxoid bacteriological agar no. 1). Selection for pantothenate prototrophs was carried out on GB1 plates supplemented with adenine (68 μg/ml), L-arginine (127 μg/ml), L-histidine (16 μg/ml), L-proline (230 μg/ml) and ampicillin (100 μg/ml). Aliquots of the transformations were plated on GB1 media supplemented with an additional 100 μg/ml of pantothenate in order to determine the number of independent viable transformants. Plates were incubated at 37 °C for 36–48 h. Longer incubation times resulted in a high frequency of revertants (10^{-3}). From about 3×10^5 transformants screened, one plasmid clone was isolated which consistently complemented the pantothenate auxotrophy of *E. coli* AT1371.

Inverse PCR

Aliquots of *Lotus* genomic DNA (5 μg) were digested to completion with each of the following restriction endonucleases: *Bam*HI, *Eco*RI, *Hin*dIII, *Not*I, *Sal*I, *Xba*I and *Xho*I. Fragments corresponding to sizes between about 2 and 15 kb were purified using the USBioClean MP kit (United States Biochemical, Cleveland, OH, U.S.A.). Each preparation (approx. 2 μg) was incubated with T_4 DNA ligase at low DNA concentration (10 ng/μl) in order to promote intramolecular circularization [22]. Reactions contained 3.0 units of T_4 DNA ligase (Boehringer-Mannheim), and the final volume was adjusted to 200 μl. Ligation proceeded overnight at 14 °C and DNA was recovered in sterile distilled water to be used as template in the following PCR step. PCR amplification was carried out using the Expand High Fidelity PCR system from Boehringer-Mannheim. Design of the *Lotus panC* specific primers Li5 (dCGGGATCCATGGTGG-GAACGAGGGCGATGAG) and Li3 (dCATCAAGCTTAT-GTATCAAAGTGCCCCAGG) followed the general strategy for inverse PCR (iPCR) described by Ochman et al. [23]. Individual PCR reactions contained, in a final volume of 50 μl, about 200 ng of re-ligated *Lotus* genomic DNA, 2.6 units of the Expand enzyme mix (*Taq* DNA polymerase and *Pwo* DNA polymerase), Expand reaction buffer (including $MgCl_2$ at a final concentration of 1.5 mM), and dNTPs and primers at final concentrations of 200 μM and 300 nM respectively. Reactions were subjected to thermal cycling (Techne PHC-3) following the manufacturer's recommendations. The iPCR product obtained was cloned using the TA cloning kit from InVitrogen NV, Leek, The Netherlands.

Production of a *Lotus panC* expression cassette

The *Lotus* ORF for PS was amplified from the original cDNA using the Expand High Fidelity PCR system from Boehringer Mannheim according to the manufacturer's protocols. The forward LC5 primer (dCGCGCTCTAGAAGGAGGAATTT-AAA**ATG**GCACCAATGGTGATATCTGAT) and the reverse LC3 primer (dGCGCGCTCGAG**TTA**CAAGTTGATTTCTA-TGTT) were designed according to the expression cassette PCR method described by MacFerrin et al. [24]. Within the LC5 and LC3 primer sequences, the *Lotus panC* start and anti-stop codons are in **bold** and the *Xba*I and *Xho*I sites are underlined. The PCR product was cloned into the pBluescript SK^- plasmid vector using restriction sites incorporated into the PCR primers. In the resulting plasmid, pSKL, the *Lotus panC* expression cassette is placed under the control of the *lac* promoter. The PCR-derived

expression cassette was sequenced, and comparison with the original cDNA sequence did not identify any mutations.

Cloning of the *O. sativum* (rice) and *Saccharomyces cerevisiae panC* genes for expression in *E. coli*

An expressed sequence tag (EST) from rice seedling roots (GenBank® accession number D25017) was identified as a putative *panC* homologue by sequence similarity searches. The corresponding cDNA clone (pBluescript SK$^+$: *Sal*I–*Not*I) was obtained from Dr. Yuzo Minobe, National Institute of Agrobiological Resources, Kannondai, Tsukuba, Ibaraki, Japan. The cloned cDNA fragment was subcloned into pBluescript KS$^-$ as a *Sal*I–*Not*I fragment and four base-pairs were deleted from the polylinker region upstream of the cDNA in order to allow expression of the encoded protein as a fusion with β-galactosidase. This was achieved by digestion with *Xho*I, removal of protruding 5′ and 3′ termini with mung-bean (*Phaseolus aureus*) nuclease, and re-ligation with T_4 DNA ligase. The resulting clone was named pRC.

The putative *Saccharomyces cerevisiae panC* gene was identified in the EMBL Nucleotide Sequence Database. A λ phage clone (λPM4950) containing a 20 kb genomic fragment of the yeast chromosome IX, which spans the putative *panC* ORF, was obtained from Dr. Carol Churcher, Sanger Centre, Hinxton Hall, Cambridge, U.K. Subcloning to express the yeast *panC* in *E. coli* was carried out in two steps. Firstly, λPM4950 DNA was digested to completion with *Hin*dIII and a 2.98 kb fragment was isolated and inserted into pBluescript. Digestion with *Eco*RV and re-ligation then generated the plasmid clone pYC (pBluescript SK$^-$: *Eco*RV–*Hin*dIII). This clone contains the yeast *panC* ORF under transcriptional control of the *lac* promoter. Since the *panC* ORF is not in-frame with *lacZ*, it is not expressed as a fusion protein.

PS activity assay

PS was assayed by coupling AMP production to the activities of myokinase, pyruvate kinase and lactate dehydrogenase as described by Pfleiderer et al. [25]. In this assay, the rate of pantothenate formation is proportional to the rate of NADH oxidation. Two molecules of NADH are oxidized for every molecule of pantothenate formed. In a final volume of 1 ml, the standard assay mixture contained 100 μmol of Tris/HCl, pH 8.0, 10 μmol of $MgSO_4$, 5 μmol of ATP (disodium salt), 1 μmol of potassium phosphoenolpyruvate, 0.36 μmol of NADH (disodium salt), myokinase (4 units), pyruvate kinase (6 units), lactate dehydrogenase (6 units), 10 μmol of β-alanine, and either 1 or 5 μmol of potassium D-pantoate. The coupling enzymes were obtained in $(NH_4)_2SO_4$ suspension (Boehringer-Mannheim). Except for pantoate, the assay components were assembled in a 1 ml UV quartz cuvette, 1–10 μl of crude extract of *E. coli* cells expressing PS, or purified PS (10 units), were added, and the volume was adjusted to 950 μl with distilled water. The absorption change at 340 nm was monitored immediately for about 2 min in order to determine background activity. The PS reaction was then initiated by addition of 50 μl potassium D-pantoate solution. The cuvette was rapidly inverted three times, and the A_{340} was monitored for another 2–4 min. Where β-alanine instead of pantoate was used to initiate the PS reaction, it was replaced by pantoate in the assay mix for background determination. Assays were carried out at 25 °C in a Perkin–Elmer spectrophotometer. PS activity was calculated using the known absorption coefficient of NADH at 340 nm (6220 $M^{-1} \cdot cm^{-1}$). All activities quoted are corrected for background activity. One unit of enzyme activity is defined as 1 nmol of pantothenate formed/min. The coupled assay was validated by omitting each of the components in turn; no activity was detected unless all the components were present. Furthermore, the assay was found to be linear up to 300 units of PS in the reaction. Kinetic measurements of the enzyme were carried out with 10 units or less of PS to ensure that the coupling enzymes were not limiting.

Purification of recombinant *Lotus japonicus* PS

E. coli AT1371 (*panC*$^-$) was transformed with the *Lotus panC*-overexpressing plasmid pSKL and grown from single colonies overnight in 10 ml of Luria–Bertani cultures containing 100 μg/ml ampicillin. Four 500 ml aliquots of 2YT (yeast/tryptone) medium containing 60 μg/ml ampicillin and 20 μg/ml isopropyl thiogalactoside in 2-litre flasks were each inoculated with 5 ml of overnight culture and incubated at 37 °C with shaking (190 rev./min) for 8–10 h before harvesting. *E. coli* cells were recovered by centrifugation and resuspended in 20 ml of buffer A (50 mM Tris/HCl/1 mM EDTA/0.1 mM dithiothreitol, pH 8.0). The cells were lysed by sonication at 0 °C. Cell debris was removed by centrifugation in a Beckman JA20 rotor (30 min, 18000 ***g***). The protein precipitating from the cleared extract in the $(NH_4)_2SO_4$ concentration interval from 30 to 40 % saturation was recovered, dissolved in 5 ml of buffer A and dialysed against 2 litres of the same buffer overnight at 4 °C. The dialysed solution was centrifuged in a Microfuge in order to remove the precipitate, and the supernatant was directly loaded on to a Pharmacia FPLC® MonoQ HR10/10 column previously equilibrated in buffer A. The column was then washed with buffer A until the A_{280} of the eluate was constant and below 0.1. A constant flow rate of 2 ml/min was maintained throughout the run. Protein was eluted in a linear gradient (80 ml) of 0–250 mM KCl in buffer A. Fractions (1 ml each) were collected throughout the gradient. The fractions were assayed for PS activity as described above, using 1 μmol of pantoate to initiate individual assays. Fractions containing PS activity were pooled, dialysed against 1 litre of buffer A overnight at 4 °C, and centrifuged to precipitate insoluble protein. Aliquots of pure PS were subjected to gel filtration on a Pharmacia Superose 6 column equilibrated in buffer A, maintaining a constant flow rate of 0.5 ml/min and collecting 1 ml fractions. Protein molecular-mass standards were chromatographed using identical conditions in order to allow estimation of the native molecular mass of PS. All standards were from the GF200 and GF1000 gel filtration kits from Sigma: Blue Dextran (2000 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), BSA (66.4 kDa), carbonic anhydrase (29.0 kDa) and cytochrome *c* (12.4 kDa).

Production of antibodies and Western-blot analysis

Antibodies against recombinant PS were generated by immunization of a rabbit, essentially as described in [26], using four injections of 100 μg of purified protein. Total soluble protein was extracted from *Lotus* leaf and root tissue in 50 mM Tris (pH 7.6)/2 mM dithiothreitol/5 mM EDTA/10 % (v/v) glycerol/broad-range protease-inhibitor cocktail (Complete™, Boehringer). Approx. 30 μg of leaf or root protein were analysed on an SDS/12 %-polyacrylamide gel and blotted on to PVDF membrane. The membrane was then challenged with either preimmune or immune serum, diluted 1:1000 in 100 mM Tris (pH 7.4)/350 mM NaCl/0.05 % Tween 20, and bound antibodies were detected with anti-rabbit IgG–alkaline phosphatase conjugate (Sigma), following the manufacturer's protocol.

RESULTS

Isolation and characterization of *Lotus japonicus* and rice cDNAs for PS

A *Lotus japonicus* cDNA for PS was isolated by functional complementation of *E. coli* AT1371, a *panC* mutant, as described in the Materials and methods section. Analysis of the nucleotide sequence of the 1.34 kb cDNA clone (pLC) revealed the presence of an ORF encoding a polypeptide of 308 amino acids (34.2 kDa). The deduced protein sequence showed 55.7 % similarity to the sequence of *E. coli* PS (GAP algorithm, GCG package), which suggested that the isolated cDNA encoded an homologous enzyme. Similarly, a rice (*Oryza sativum*) EST clone with sequence similarity to *E. coli panC* was sequenced completely. The 1.26 kb cDNA contains an ORF encoding a polypeptide of 313 amino acids (33.9 kDa) that is 65.3 % similar to *Lotus* PS and 50.9 % similar to the enzyme from *E. coli*. Given their similarity to *E. coli panC*, the cDNAs were designated *Lotus japonicus panC* and *Oryza sativum panC*. The *panC* genes from *E. coli* [7] and *Saccharomyces cerevisiae* (EMBL accession number Z38059), rice and *Lotus* (the present study) were all able to complement the PS lesion in the *E.coli panC* mutant AT1371 (see below), confirming that they encode the enzyme. An alignment of the corresponding deduced amino acid sequences is shown in Figure 1(A). The yeast sequence is the least similar (46 % identity with *Lotus* and *E. coli* and 41 % with rice *panC*) and appears to be longer at the *N*-terminus by some 30 amino acids. The proteins are quite similar throughout their length, although there are two regions of greater conservation, one near the N-terminus and one in the middle of the protein. However, there is no clear indication of any functional significance in these regions, for example corresponding to an ATP-binding site. Searching the EMBL/GenBank® databases identified further homologues to *E. coli panC*, which were omitted from the protein sequence alignment in Figure 1(A) for clarity. These putative *panC* genes from the fission yeast *Schizosaccharomyces pombe* (accession number Z49811), *Bacillus subtilis* (L47709), *Mycobacterium tuberculosis* (Z95557), *Synechocystis* sp. (U44896), and *Helicobacter pylori* (AE000523) are respectively 52, 51, 49, 48 and 44 % similar, at the amino acid level, to *Lotus* PS. The similarities between all database sequences for PS is shown graphically in the phylogram in Figure 1(B) (constructed with the clustalW and Phylip packages). Since there is no accepted ancestral sequence, it is not possible to draw a firm conclusion from this diagram, but it is noteworthy that the two higher-plant sequences appear to be more similar to PS from yeast and *E. coli* than to the cyanobacterial sequence.

The *panC* ORFs of both *Lotus* and rice start with the first ATG codons present in the respective cDNAs, and align with the start of the *E. coli* protein (Figure 1A). These putative translation start sites are in good agreement with the published consensus sequences for translation initiation in dicotyledonous and monocotyledonous plants [27,28] respectively. However, it could not be said with confidence that the *panC* ORFs of *Lotus* and rice are complete at the 5′ end. To check this, a rice cDNA library from etiolated shoots was obtained (Clontech, Palo Alto, CA, U.S.A.) and subjected to one-armed PCR in order to isolate a more complete 5′ portion of the *panC* cDNA. The presence of *panC* cDNAs in the library was shown by the use of internal primers, but no extra 5′ sequence was obtained (results not shown). The original *Lotus* λZAP cDNA library was then re-screened by nucleic acid hybridization. Out of $> 10^5$ plaques screened, one was identified that positively hybridized to a *Lotus panC* probe. However, the newly isolated clone contained no extra 5′ sequence (results not shown). These data support the conclusion that the original cDNAs are essentially full-length, although it is possible that the low abundance of *panC* in the libraries may have prevented the isolation of longer clones.

Isolation of *Lotus japonicus* genomic flanking regions of *panC* and genomic Southern analysis

As an alternative approach to determine whether the cDNAs encoded full-length proteins, flanking regions of the *panC* gene were isolated from *Lotus japonicus* genomic DNA by inverse PCR. The binding sites of the iPCR primers on the *panC* gene were chosen in order to generate sufficient overlap of cDNA and genomic sequence. The 'inverse' genomic DNA preparation generated with *Bam*HI was the only effective template, giving rise to an iPCR product that contains 5′ and 3′ flanking regions separated by a unique *Bam*HI site. A diagram of the cloned iPCR product is given in Figure 2(A). The DNA sequence of the insert (Figure 2B) includes matches identical with the 5′ and 3′ ends of the *Lotus panC* cDNA, confirming that true *panC* flanking sequences were amplified. In the 5′ flanking region there is a stop codon 24 bases upstream from the putative initiation site, which is in-frame with the *panC* ORF. If the full-length *panC* transcript is longer than the cDNA, then the presence of this stop codon means that the ORF must nevertheless start from the ATG previously identified (position 670 in Figure 2B). The only possible exception is if there is an intron in this region. Prediction of splice sites at the NetPlantGene Server (Centre for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark) [29], a network trained on *Arabidopsis thaliana* (thale cress) sequences, yielded possible donor splice sites at positions 258 and 281, and possible acceptor splice sites at positions 179, 316, 338 and 536 in the sequence given in Figure 2(B). However, no intron–exon borders were predicted between the stop codon (651) and the start of the cDNA (670), nor within the coding sequence.

To investigate the copy number of the PS gene, a Southern blot was prepared of *Lotus* genomic DNA digested with a range of restriction enzymes. The blot was probed with the complete 1.34 kb cDNA for *Lotus panC*, radiolabelled with ^{32}P, followed by autoradiography. It is clear from Figure 3 that a simple restriction pattern is obtained with the majority of restriction digests, suggesting that the *panC* gene is single copy. The cDNA has internal sites for *Stu*I and *Xba*I, but none of the other enzymes, so presumably these sites are present in introns. The *Sma*I digest was unsuccessful, so that the majority of the DNA remained at the top of the gel, while the *Eco*RI digest appears to be incomplete, since the intensity of the bands was much less in this track than in the others.

We also attempted to carry out Northern-blot analysis of *Lotus* RNA probed with the radiolabelled cDNA. However, we were unable to detect a reproducible signal, although the same blot was used successfully for other *Lotus* clones (C. A. Powell and A. G. Smith, unpublished work). This indicates that the *panC* message is in low abundance.

Functional complementation of the *E. coli panC* allele and expression of PSs in *E. coli*

The *panC* genes from *Lotus*, rice and yeast were all checked for their ability to complement the *panC* phenotype of *E. coli* AT1371. A plasmid clone of the *E. coli panC* gene (pCC) obtained from Dr. A. Lewendon, Department of Biochemistry, University of Leicester, Leicester, U.K., served as a positive control. The *E. coli* mutant was transformed with the relevant

A

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lotus  ---------------------------------MAPMVISDKDEMRKWSRSMRSQGKL-
rice   ---------------------------------MAAPREPEVIRDKAAMRAWSRRRRAEGKT-
coli   ------------------------------------MLIIETLPLLRQQIRRLRMEGKR-
yeast  MNISRCSVHIYVYKKWCVLILHCFIQVFILHIHIELMKIFHTVEEVVQWRTQELRETRFR

lotus  --IALVPTMGFLHEGHLSLVRDAHNHAD----LVAVSIYVNPGQFSPTEDLSAYPSDFQG
rice   --VAVVPTMGYLHQGHLSLISAAAAAASADPVAIVVTIYVNPSQFAPSEDLATYPSDFAG
coli   --VALVPTMGNLHDGHMKLVDEAKARAD----VVVVSIFVNPMQFDRPEDLARYPRTLQE
yeast  ETIGFVPTMGCLHSGHASLISQSV----KENTYTVVSIFVNPSQFAPTEDLDNYPRTLPD

lotus  DLQKLMSVPGGVDVVFHPHNLYDYGGDGGDAVAECGGDGVVSCVDRRSGFGHETWVRAEK
rice   DLRKLAST-GVVDAVFNPPDLYVRGAGRRGA----GSGGAISCLEEAAGDGHETWVRVER
coli   DCEKLNKRK--VDLVFAPSVKEIY----------PNGTETHTYVDVPG
yeast  DIKLLESLK--VDVLFAPNAHVMY----------PQGIPLDIEEQKG----PFVSVLG

lotus  LEKPLCGKSRPVFFRGVATIVTKLFNIVEPDVAVFGKKDYQQWKIIQRMVRDLDFSIKVI
rice   LEKGLCGASRPVFFRGVATIVSKLFNIIEPDVPVFGKKDYQQWRVILPYWSGLDFGIEIM
coli   LSTMLEGASRPGHFRGVSTIVSKLFNLVQPDIACFGEKDFQQLALIRKMVADMGFDIEIV
yeast  LSEKLEGKTRPNFFRGVATVVTKLFNIVMADVAYFGQKDIQQFIVLQCMVDELFVNTRLQ

lotus  GSEVIREKDGLAMSSRNVYLSPEEREKAVSINKSLFRAKSA----AEDGQIHCEKLINLV
rice   GSRNCARTDGLAMNSRNVHLSREEGKKALSISRSLVDARTG----ALKGNTDSKQIKNKI
coli   GVPIMRAKDGLALSSRNGYLTAEQRKIAPGLYKVLSSIADK----LQAGERDLDEIITIA
yeast  MMPIVRNNNGLALSSRNKYLCPESLKISENLYRGLKAAENAIRRLAPGGRLSRSEIIDTV

lotus  VQ----SITEAGGRIDYAEIVDQNNLEKVEWIKG-----PVVFCVSAWFGK-------AR
rice   VQ----TLTETGGQVDYVEIVEQESLVPVEQIDG-----PVVICVAAWFGK-------VR
coli   GQ----ELNEKGFRADDIQIRDADTLLEVSETSK-----RAVILVAAWLGD-------AR
yeast  TQIWAPYVDSHDFKIDYVSLADFKTLDELSDVENTSEQQPIVISCAVYVTDREKPDTVVR

lotus  LIDNIEINL--
rice   LIDNIEIDTRS
coli   LIDNKMVELA-
yeast  LIDNIVI----
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B

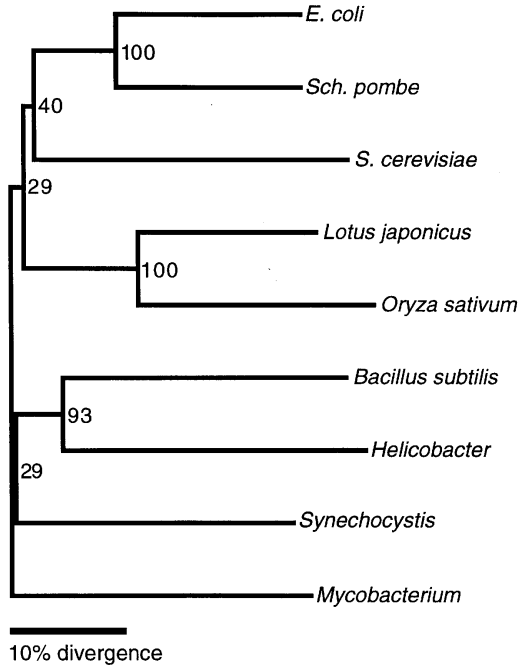

Figure 1 Alignment of PS amino acid sequences from *Lotus*, rice, yeast and *E. coli*, and phylogeny of known PS sequences

(**A**) The protein-sequence alignment was created with clustalW (v1.5) multiple sequence alignment software. Using the GAP algorithm (GCG package), percentage similarities to the *L. japonicus* (lotus) enzyme are 65, 56 and 46% for PS protein sequences from *Oryza sativa* (rice), *E. coli* (coli), and *S. cerevisiae* (yeast) respectively. (**B**) The phylogram shown was created from an alignment of all known PS sequences using clustalW (v1.5) and Phylip Bootstrap software. Alignment positions with gaps were excluded from the analysis. Percentage confidence values for individual nodes were obtained from Bootstrap trials ($N = 1000$). See the text for the database accession numbers of individual PS sequences. There are two entries for *panC* from *Synechocystis* in the database: U44896, which encodes a protein of 275 amino acids similar to the size of all other known PSs, and D90912, which encodes a protein of 513 amino acids, only the *N*-terminus of which corresponds to PS from other organisms. In this phylogenetic analysis we have used that part of the *Synechocystis* PS sequence that is identical in both database entries, that is, the N-terminal 211 amino acids.

plasmids and streaked on to GB1 plates with or without pantothenate as described in the Materials and methods section for the isolation of the *Lotus panC* cDNA (Figure 4). The *panC* clones from *Lotus*, rice and yeast, as well as *E. coli*, all complemented the PS lesion in *E. coli* AT1371, whereas pBluescript did not. Although yeast PS shows a lower degree of sequence similarity to *E. coli* PS than is the case for PS from *Lotus* or rice, the yeast clone was slightly more effective at rescuing the mutant than the plant clones. Each of these strains was also grown in 2YT culture medium, and cleared extracts from the cells were assayed for PS activity using the coupled assay in the Materials and methods section (Table 1). The levels of PS activity observed in *E. coli* strains AT1371 and K12 are in accord with previous reports [5,25]. PS activity could be demonstrated readily in extracts from cells transformed with the *E. coli* (pCC) or yeast (pYC) *panC* genes. However, with the *Lotus* (pLC) or rice (pRC) cDNAs there was no detectable activity, indicating that the level of activity necessary for functional complementation was very low. Therefore, a *Lotus panC* clone (pSKL) for high-level expression of the encoded enzyme was constructed by expression-cassette PCR as described in the Materials and methods section. When the *E. coli* mutant was transformed with this clone, PS activity in the extract was detected using the standard assay procedure, albeit at low levels, comparable with that seen in wild-type *E. coli*. Intriguingly, this activity was 3–4-fold higher when pantoate was used at a concentration of 1 mM instead of 5 mM.

The lactone form of pantoate, pantoyl-lactone, is not a substrate of purified *E. coli* PS [9]. When pantoyl-lactone was substituted for pantoate, some residual activity ($< 1\%$) was detected in AT1371::pCC, which is likely to be due to a hydrolytic activity present in the *E. coli* extracts that converts pantoyl-lactone into pantoate. Similarly, pantoyl-lactone was not an effective substrate for PS from either yeast or *Lotus* (Table 1).

Characterization of recombinant *Lotus japonicus* PS

Recombinant *Lotus* PS was overexpressed in *E. coli* and purified by $(NH_4)_2SO_4$ fractionation and anion-exchange chromatography at a yield of about 9 mg per litre of *E. coli* culture. The freshly prepared enzyme was approx. 95% pure, as judged by SDS/PAGE and had a specific activity of 1360 units/mg when assayed at a pantoate concentration of 1 mM. It was eluted in a single peak from a Superose 6 gel-filtration column. However, no increase in specific activity over the anion-exchange-chromatography step was achieved. Pure *Lotus* PS in purification buffer was stable at -20 °C. When stored at 4 °C it lost about 25% activity within 4 weeks. The enzyme required D-pantoate and β-alanine for activity as was reported for PS from *E. coli* [9,25]. Specifically, no activity could be detected when pantoate was replaced by pantoyl-lactone or oxopantoate, in accordance with the assays on *Lotus* PS in crude *E. coli* extracts (see the preceding subsection). The pH optimum for purified *Lotus* PS was determined using 100 mM Tris/HCl or 100 mM potassium phosphate buffers (pH 6–9) in the coupled PS assay. Optimal activity was found at pH 7.8 in Tris/HCl. Activity decreased sharply towards a more acidic pH in Tris/HCl and was nil at pH 7.0, whereas there was only a slight decrease towards higher pH, with

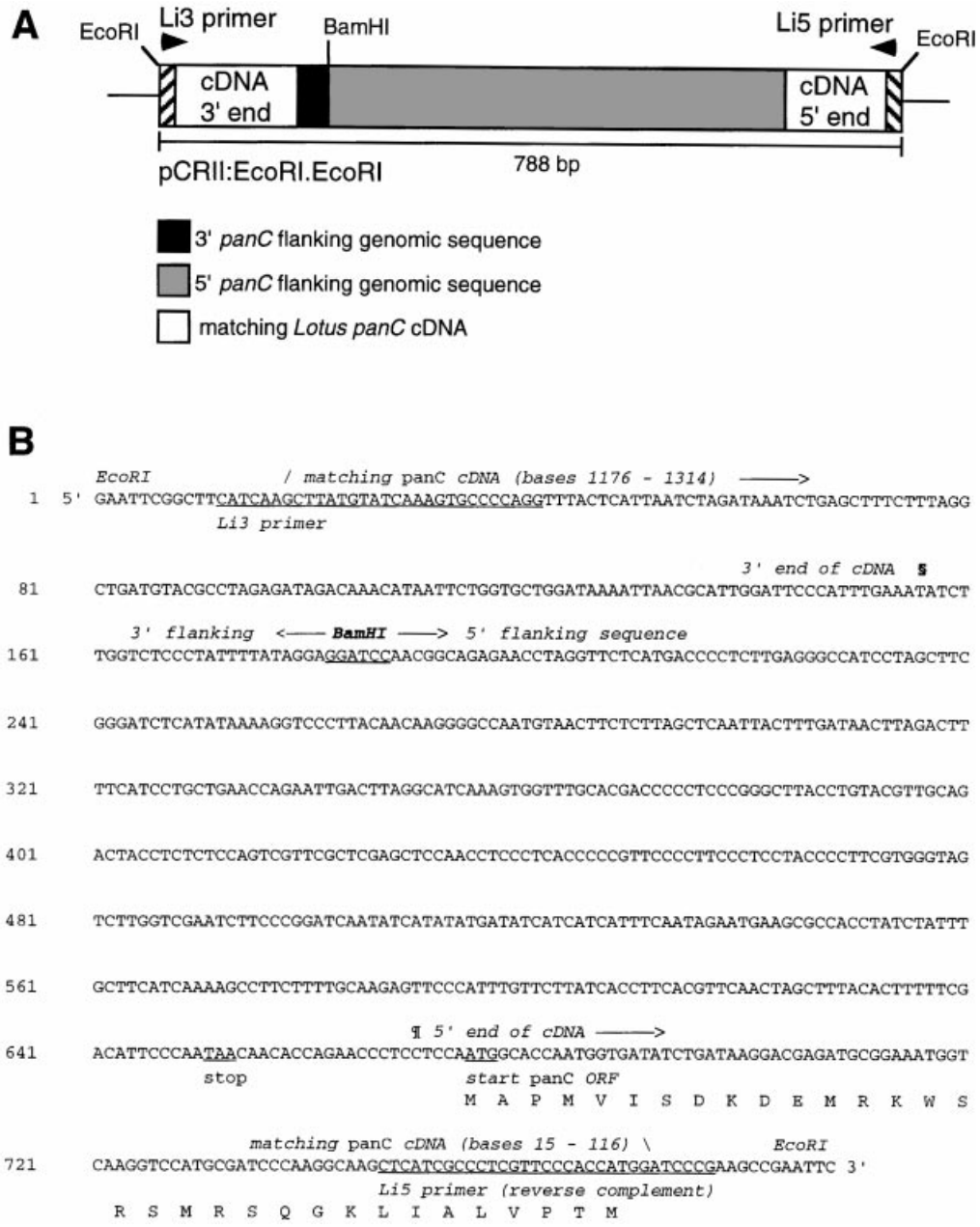

Figure 2 Genomic sequences flanking the *panC* gene in *Lotus japonicus*

(**A**) Schematic representation of the iPCR product cloned into the TA cloning vector pCRII (InVitrogen). *Lotus* genomic DNA was digested with *Bam*HI, re-ligated and used as template in the PCR reactions. Therefore, the 5′- and 3′-flanking sequences are adjacent in the iPCR product, separated by a unique *Bam*HI recognition site. (**B**). Nucleotide sequence of the cloned iPCR product. The indicated matches with the *Lotus panC* cDNA mean identical sequences. Positions corresponding to the first base (5′–¶) or the last base (3′–§) of the *panC* cDNA are marked. Within the 5′ flanking genomic sequence there is a stop codon in-frame with the *panC* ORF. Assuming there are no intron–exon borders between this stop codon and the proposed translation start site, the *Lotus panC* ORF would appear to be complete.

about 75% activity left at pH 9.0. In potassium phosphate, optimal PS activity was observed at pH 8.0, with 50% of that activity left at pH 7 and 15% left at pH 6 and pH 9 respectively. Optimal activity in Tris/HCl was almost twice that in potassium phosphate. Given the likely location of the enzyme in the plant cell cytosol, which has a pH of about neutral, the effect of pH on recombinant PS *in vitro* is perhaps surprising, although, *in vivo*, other factors may influence the activity. In addition, the pH response *in vitro* may simply reflect the optimum for the operation of the coupled assay.

Analysis of the purified *Lotus* PS by SDS/PAGE revealed a single polypeptide of approx. 34 kDa (results not shown), which agrees well with the calculated subunit molecular mass of 34 240 Da. The native molecular mass of recombinant *Lotus* PS was estimated to be 72.8 kDa by gel filtration on Superose 6 (results not shown), suggesting a homodimeric structure for the enzyme. *N*-terminal sequencing of the recombinant *Lotus* PS revealed a primary peptide sequence of PMVISDKDEMRKWSR and a less abundant sequence of APMVISDKDEMRKWS. Comparison with the predicted protein sequence (Figure 1A) shows that

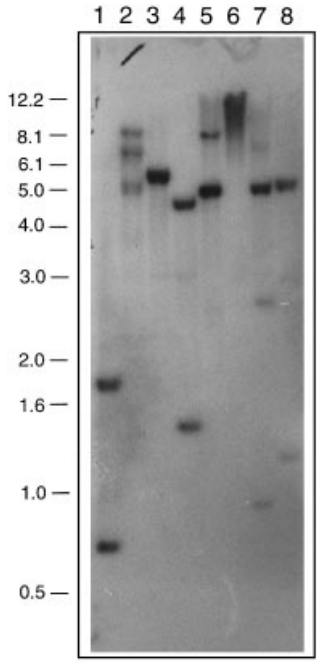

Figure 3 Southern-blot analysis of genomic DNA from *Lotus*, probed with *panC*

A 5 μg portion of *Lotus* genomic DNA was digested for 2 h with different restriction enzymes, electrophoresed on a 1%-agarose gel, and then blotted on to nylon membrane. The blot was probed with the 1.34 kb insert from pLC radiolabelled with [^{32}P]dCTP, washed as described in [19] and then autoradiographed for 5 days at −70 °C with an intensifying screen. Lane 1, *Dra*I; lane 2, *Eco*RI; lane 3, *Eco* RV; lane 4, *Hin*dIII; lane 5, *Pst*I; lane 6, *Sma*I; lane 7, *Stu*I; lane 8, *Xba* I. The values (in kb) on the left are those of molecular-size markers.

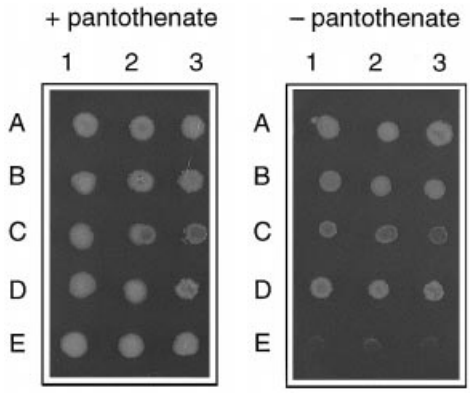

Figure 4 Functional complementation of the PS-deficient *E. coli panC* mutant

E. coli AT1371 (*panC*$^-$) cells were transformed with plasmids pCL, pYC, pLC and pRC, which contain the cloned *panC* genes from *E. coli* (A), yeast (B), *Lotus* (C), and rice (D) respectively or with pBluescript (E). The cells were grown in Luria–Bertani medium with ampicillin, harvested by centrifugation and resuspended in GB1 minimal medium to give an attenuance (D_{600}) of 0.1. Aliquots (2 μl) of cell suspensions A–E were applied on to GB1 minimal plates containing 100 μg/ml pantothenate (+ pantothenate) or lacking the vitamin (− pantothenate) in lane 1. Ten- and hundred-fold dilutions of the original suspensions were applied in lanes 2 and 3 respectively. The photograph was taken after 36 h of incubation at 37 °C.

the majority of the recombinant protein had lost the two N-terminal residues (Met^1 and Ala^2), whereas a smaller proportion had lost just the first methionine residue.

Kinetic constants of recombinant PS

The steady-state kinetics of purified recombinant *Lotus* PS were investigated using the coupled assay described in the Materials and methods section. The Michaelis plot in Figure 5(a) reveals normal saturation kinetics for β-alanine, whereas pantoate inhibited the enzyme at concentrations above about 0.5 mM. Kinetic constants for pantoate are therefore based on a restricted range of pantoate concentrations (0.004–0.2 mM) where substrate inhibition was negligible and Michaelis–Menten kinetics were obeyed [30]. The K_{m*app} for pantoate at saturating β-alanine concentration as derived from the linearized plot in Figure 5(b) is 44 μM. V_{max} in this plot is 1098 units/mg. Assuming a subunit mass of 34 kDa, this is equivalent to a k_{cat} of 0.62 s^{-1}. The K_{m*app} for β-alanine depends on the pantoate concentration and is 441 μM at 20 mM pantoate or 986 μM at 0.5 mM pantoate (Figure 5c). However, the ratio of V_{max}/K_{m*app} for β-alanine remains essentially unchanged. This pattern implies pantoate is an uncompetitive inhibitor [31]. The simplest model of this inhibition type assumes that a second substrate molecule binds to the enzyme–substrate complex to form a catalytically inert ES_2 species. The corresponding catalytic scheme for uncompetitive substrate inhibition [30,31] (a) and the corresponding rate law used in the fit (K_S and K'_S are the dissociation constants for the first and second substrate molecules respectively) are given below:

(a)
$$E + S \underset{}{\overset{k_S}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$
$$\Big\downarrow\Big\uparrow S, k'_S$$
$$ES_2$$

(b)
$$v = \frac{v_{max}[S]}{k_S + [S](1 + [S]/k'_S)}$$

Fitting the rate law for uncompetitive substrate inhibition to the observed activity data demonstrates this model can predict the actual behaviour of *Lotus* PS very well (Figure 6). Values derived for K_S and K'_S, the dissociation constants for the first and second substrate molecule, are 42 ± 2 μM and 5.33 ± 0.34 mM respectively, that is, the second pantoate molecule binds with more-than-100-fold lower affinity than the first. The V_{max} in this fit is equivalent to a k_{cat} of 0.63 ± 0.1 s^{-1}, which is practically identical with the value taken from the linearized plot in Figure 5(b). The dissociation and Michaelis constants for pantoate, K_S and K_{m*app}, are also nearly identical, which suggests that binding of the first pantoate molecule is fast compared with the subsequent steps of the catalytic cycle.

We investigated whether there was any potential feedback or feedforward regulation of *Lotus* PS by testing the effect of intermediates of pantothenate biosynthesis, various acyl-CoA compounds and fatty acids on the activity of recombinant *Lotus* PS. Pantoate and β-alanine were present at 0.1 mM and 1 mM, values close to the respective K_{m*app} constants. However, none of the compounds had a pronounced effect; in particular, there was no product inhibition of PS by pantothenate and no effect with oxopantoate. An appreciably altered PS activity occurred only with palmitic acid (16:0), which, at a concentration of 2 μM, increased activity by one-third.

Western analysis of PS in *Lotus* tissues

As mentioned in the Introduction, it has not been possible to demonstrate the activity of PS, nor any of the other enzymes of pantothenate biosynthesis, in plant tissues, presumably owing to the low levels of these enzymes. We therefore took advantage of

Table 1 Expression of PSs from *E. coli*, yeast, rice and *Lotus* in a PS-deficient *E. coli* mutant (AT1371)

PS was assayed in crude extracts as described in the Materials and methods section. One unit of activity corresponds to 1 nmol of pantothenate formed/min; –, not determined.

E. coli strain	Vector	Specific activity (units/mg) With 5 μmol of pantoate	With 5 μmol of pantoyl-lactone
K12 (wild type)	None	13.4	–
AT1371 (*panC*$^-$)	pBluescript	Not detectable	
AT1371 (*panC*$^-$)	pCC *E. coli panC*	957.4	6.0
AT1371 (*panC*$^-$)	pYC yeast *panC*	88.0	3.1
AT1371 (*panC*$^-$)	pRC rice *panC-lacZ* fusion	Not detectable	–
AT1371 (*panC*$^-$)	pLC *Lotus panC-lacZ* fusion	Not detectable	–
AT1371 (*panC*$^-$)	pSKL *Lotus panC* expression cassette	8.7	Not detectable

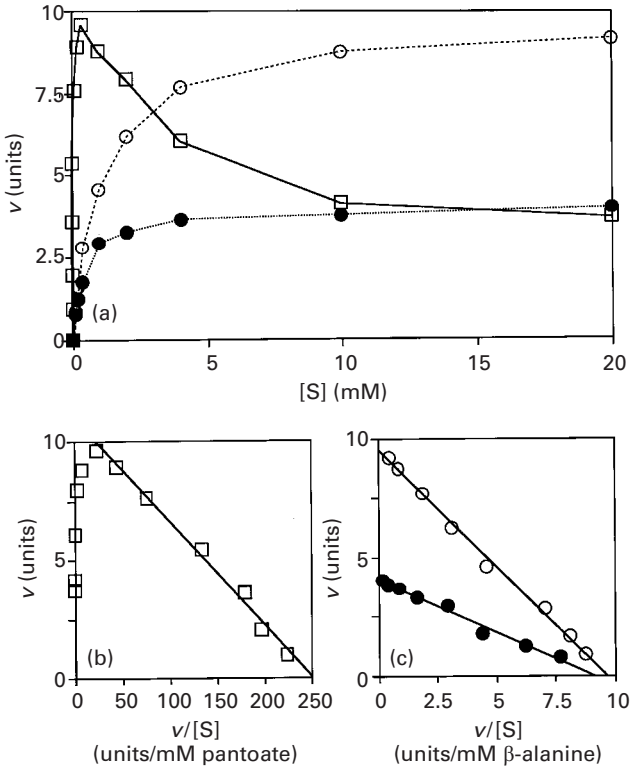

Figure 5 Steady-state kinetic analysis of *Lotus* PS

(**a**) Michaelis–Menten plot of PS activity as a function of pantoate concentration (□) at saturating β-alanine concentration (20 mM) or as a function of β-alanine concentration at constant pantoate concentrations of 0.5 mM (○) or 20 mM (●). The response of the enzyme to pantoate does not follow simple saturation kinetics, with concentrations above 0.4 mM inhibiting activity. However, Michaelis–Menten kinetics seem to be obeyed for the second substrate, β-alanine. PS was assayed in aliquots of 10 μg of enzyme as described in the Materials and methods section. (**b**) Linearized plot (Eadie–Hofstee) of PS activity as a function of pantoate concentration (□). K_{m^*app} for pantoate derived from the linear range of the plot is 44 μM, and k_{cat} is 0.62 s^{-1}. (**c**) Eadie–Hofstee plot of PS activity as a function of β-alanine concentration. K_{m^*app} values for β-alanine are 986 μM at 0.5 mM pantoate (○) and 441 μM at 20 mM pantoate (●). While K_{m^*app} for β-alanine is strongly affected by pantoate, k_{cat} over K_{m^*app} remains nearly constant, equalling 0.55 and 0.53 mM$^{-1}\cdot$s^{-1} at 0.5 and 20 mM pantoate respectively. This pattern is indicative of uncompetitive inhibition by pantoate.

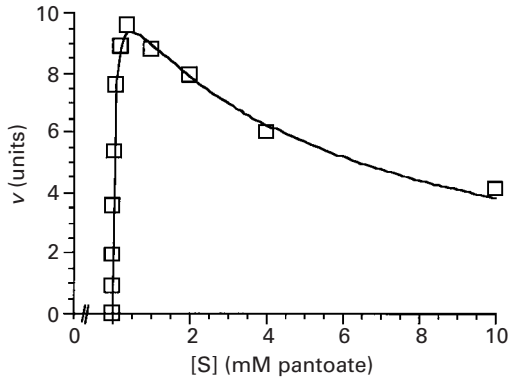

Figure 6 Substrate inhibition of *Lotus japonicus* PS

The Figure shows a fit of the rate law for uncompetitive substrate inhibition to the PS activity data in Figure 5(a) (□). The fit was carried out using Kaleidagraph software and spans a pantoate concentration interval from 0 to 10 mM.

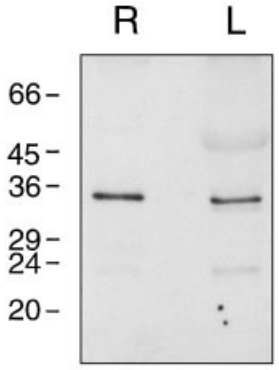

Figure 7 Western-blot analysis of PS in *Lotus* tissues

A 30 μg portion of soluble protein from root and leaf tissue from 8-week-old *Lotus* plants were subjected to SDS/PAGE, transferred to PVDF membrane, and then challenged with antiserum raised against recombinant *Lotus* PS protein. Bound antibodies were detected with anti-rabbit IgG-conjugated alkaline phosphatase. R, root sample; L, leaf sample. The values on the left (kDa) relate to molecular-mass markers.

the purified PS protein to raise polyclonal antibodies in a rabbit for use in Western-blot analysis. The antiserum was able to detect a band of 34 kDa in soluble protein extracts of *E. coli* AT1371 harbouring pSKL, whereas no cross-reaction was observed with preimmune serum (results not shown). Accordingly, the antiserum was used to challenge a Western blot of soluble proteins from *Lotus* leaf and root tissue (Figure 7). Again

a single band of 34 kDa was detected in both samples, demonstrating that the protein is expressed in roots and leaves, essentially to the same extent.

DISCUSSION

We have shown that cDNA clones from *Lotus japonicus* and rice encode functional PS (Figure 4) and that the protein is present in both root and leaf tissue of *Lotus* (Figure 7). This provides the first conclusive evidence for the pantothenate biosynthesis pathway in plants. Given the ability of PS from yeast, rice and *Lotus* to complement an *E. coli* mutant lacking PS, the enzyme seems to be conserved across phylogenetically distant organisms. Pantothenate is the essential precursor to CoA which is required as a cofactor by a large number of enzymes. It seems probable, therefore, that pantothenate biosynthesis evolved comparatively early.

We are confident that the *Lotus* and rice cDNAs are full-length, as the *N*-termini of the encoded proteins align approximately with that of *E. coli* PS (Figure 1A), and analysis of the *Lotus panC* 5′ flanking genomic sequence found an in-frame stop codon 24 bases upstream of the proposed initiating ATG (Figure 2B). Furthermore, the *N*-terminal parts of *Lotus* and rice PS do not conform to the criteria for targeting peptides [32] and so are unlikely to be able to target the protein to the chloroplasts or mitochondria. From this we infer that the proteins encoded by the *Lotus* and rice cDNAs are cytosolic enzymes. This conclusion is supported by the fact that the higher-plant PSs are less similar to *panC* from the cyanobacterium *Synechocystis* (48 %) than from *E. coli* (56 %). It would be expected that, if the higher-plant enzymes were located in the chloroplast, they would be closest to the *Synechocystis* sequence, since chloroplasts arose from the endosymbiosis of prokaryotic algae very similar to cyanobacteria [33]. Interestingly there does appear to be an *N*-terminal extension (of about 30 amino acids) on PS from budding yeast (*S. cerevisiae*) (Figure 1A), which has some characteristics of a mitochondrial transit peptide [32]. The *S. cerevisiae* sequence for OPHMT (*panB*) also has an *N*-terminal extension compared with the enzyme from *E. coli* (U. Genschel, unpublished work). If these are indeed mitochondrial transit peptides, it would seem that pantothenate is synthesized in the yeast mitochondrion. In contrast, neither the *panB* nor the *panC* sequences from fission yeast (*S. pombe*) have *N*-terminal extensions, suggesting that the enzymes are cytosolic in this organism.

Recombinant *Lotus* PS was overexpressed in *E. coli* and purified from the soluble fraction. In common with the *E. coli* enzyme, *Lotus* PS has a homodimeric subunit structure. Another shared feature of both enzymes is a much higher affinity for pantoate than for β-alanine. However, previous studies of bacterial PS have consistently found hyberbolic substrate binding curves for all substrates [9,25]. This is not the case for *Lotus* PS, where allosteric binding of a second molecule of pantoate leads to uncompetitive inhibition (Figure 6). In contrast with the substrate inhibition, a number of related compounds which were tested had little or no effect on PS activity. Although pantothenate biosynthesis is assumed to proceed largely unregulated in *E. coli* [5], it is likely that corresponding pathway in plants is more finely tuned to the actual requirements of the cell. Indeed, pantothenate kinase, which catalyses the next step in the pathway of CoA biosynthesis, is activated four-fold in germinating spinach (*Spinacia oleracea*) seeds compared with mature leaf tissue [34]. It is possible, therefore, that pantoate inhibition of *Lotus* PS may function to limit pantothenate production *in vivo*.

The specificity of *Lotus* PS for pantoate rather than pantoyl-lactone, and its likely location in the cytosol, is in contradiction to a report on pantothenate biosynthesis in spinach [35], which reported the detection of PS activity in the chloroplast stroma. This activity could only be demonstrated with pantoyl-lactone. It is possible therefore, that, in higher plants, two biosynthetic pathways to pantothenate may proceed independently via the acid and lactone forms of pantoate in the cytosol and in the plastids respectively. Although the genomic Southern blot of Lotus DNA (Figure 3) indicated that there was a single gene encoding the cDNA clone, it is possible that there is another gene with insufficient sequence similarity for the two to cross-hybridize. However, PS from *Lotus*, yeast and *E. coli* all use pantoate in preference to the pantoyl-lactone (Table 1; [9]), and so if the activity in spinach is genuinely involved in pantothenate biosynthesis, it must be quite unlike the previously characterized enzymes. The antibodies against PS have demonstrated its presence in both root and leaf tissue (Figure 7). They will be a useful tool to establish the location of pantothenate biosynthesis within the plant cell.

The isolation of a *L. japonicus* cDNA for PS has shown that at least the last step in the biosynthesis of pantothenate is the same in plants as in *E. coli*. Using a similar strategy we are now aiming to isolate further plant genes on the pantothenate-biosynthetic pathway.

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