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Cloning and expression of homospermidine synthase from *Senecio vulgaris*: a revision

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Abstract

Homospermidine synthase, which catalyses the first pathway-specific reaction in pyrrolizidine alkaloid biosynthesis, was cloned from root cultures of *Senecio vulgaris* and expressed in *E. coli*. The open reading frame encodes a protein of 370 amino acids with a molecular mass of 40,740 Da. The enzyme is strictly dependent on spermidine as aminobutyl donor since it cannot be substituted by putrescine. The homospermidine synthase from *S. vulgaris* showed 97.9 and 99.3% nucleic acid identity with two HSS sequences from the closely related species *Senecio vernalis*. This report also revises data from a previous publication (Kaiser, A., 1999. Cloning and expression of a cDNA encoding homospermidine synthase from *Senecio vulgaris* (Asteraceae) in *Escherichia coli*. Plant J. 19, 195–201.) that is incorrect. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Homospermidine synthase; *Senecio vulgaris*; *Senecio vernalis*; Pyrrolizidine alkaloids; Deoxyhypusine synthase; Spermidine dependency

1. Introduction

Recently the cloning and expression of a cDNA encoding homospermidine synthase of *Senecio vulgaris* (Asteraceae) was published (Kaiser, 1999).[1] The published results need to be revised, due to incorrect sequencing.

Homospermidine synthase catalyzes the first pathway-specific reaction in the biosynthesis of pyrrolizidine alkaloids (PAs), a class of typical constitutive plant secondary defensive compounds which are being studied in our lab since 15 years (Hartmann and Witte, 1995; Hartmann, 1999; Hartmann and Ober, 2000). Root cultures and plants of *S. vulgaris* and the taxonomically closely related species *S. vernalis* (Kadereit, 1984) proved to be excellent systems to study the physiology and biochemistry of PA biosynthesis (Böttcher et al., 1993, 1994; Hartmann and Dierich, 1998), transport (Hartmann et al., 1989) and vacuolar storage (Ehmke et al., 1988). Recently we succeeded in purifying homospermidine synthase from root cultures of *S. vernalis* to homogeneity and yielded four peptides by microsequencing. Two of them showed homology in database searches to deoxyhypusine synthase of yeast and *Neurospora crassa*. Using all available cDNA sequences of deoxyhypusine synthase we created an alignment to construct degenerate primers against highly conserved parts of the sequences. With these primers we were able to identify the cDNA sequences for deoxyhypusine synthase and homospermidine synthase of *S. vernalis* (Ober and Hartmann, 1999b). Because of the close relationship between *S. vernalis* and *S. vulgaris* we used the gene specific primers generated for 3′- and 5′-RACE of the *S. vernalis* homospermidine synthase (Ober and Hartmann, 1999b) to identify the corresponding gene in *S. vulgaris*. The resulting sequence information was used to generate a full-length cDNA clone, which was sequenced and overexpressed in *Escherichia coli* for further characterization.

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[1] The paper was published under the name of our institute and without knowledge and permission of the senior author. It presented incomplete and partly wrong results and did not consider basic scientific input of other scientists involved in the study. We submitted a brief manuscript for publication in *The Plant Journal* that would have set the record straight in this matter. Unfortunately *The Plant Journal* is unable to consider such a "letter" type of communication for publication.

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2. Results and discussion

2.1. DNA sequence analysis

The full-length cDNA encoding HSS of *S. vulgaris* contains an open reading frame (ORF) of 370 amino acids with a molecular mass of 40,740 Da (EMBL database accession no. AJ251500). The 685 and 535 bp fragments generated by 5′- and 3′-RACE techniques revealed untranslatable regions of 44 bp at the 5′-end and of 140 bp at the 3′-end of the sequence. Comparing the nucleic acid sequence of the full-length cDNA generated by *pfu*-DNA polymerase with the overlapping sequence of the DNA fragments generated by RACE techniques, the 3′-end-containing fragment showed sequence identity, while the 5′-end-containing fragment showed over a stretch of 471 bp base exchanges at 6 positions, which resulted in the amino acid sequence in only one conservative exchange, indicating that more than one gene for HSS is expressed in roots of *S. vulgaris*, as it was already suggested for HSS of *S. vernalis* roots (Ober and Hartmann, 1999b).

2.2. cDNA sequence comparison for homospermidine synthase of S. vulgaris and S. vernalis

Recently we succeeded in identifying a second cDNA sequence encoding homospermidine synthase in roots of *S. vernalis* (unpublished results). The two sequences from *S. vernalis* share identities of 98.1% on the nucleic acid level and 99.7% on amino acid level, respectively. The comparison of the two sequences with the cDNA sequence encoding homospermidine synthase of *S. vulgaris* revealed a sequence identity 97.9 and 99.3% (nucleic acid level) and 99.4 and 99.7% (amino acid level), respectively. Thus, the cDNA sequences obtained from the two *Senecio* species are as similar as the two sequences found in the roots of *S. vernalis*, reflecting the close genetic relationship between these two species. An alignment of all known amino acid sequences of HSS and DHS of plant origin is shown in Fig. 1.

2.3. Expression of recombinant HSS from S. vulgaris in E. coli

SDS-PAGE of *E. coli* BL21(DE3) expressing recombinant HSS cDNA from *S. vulgaris* (Fig. 2) revealed a protein of approximately 45 kDa subunit size. This size is well in accordance with the electrophoretic properties of HSS purified from root tissue of *S. vernalis*. The latter consists of four identical subunits with an native molecular weight of approximately 174 kDa (Ober and Hartmann, 1999b). Assays of the recombinant *S. vulgaris* protein for HSS and DHS activity revealed only HSS activity but no DHS activity (data not shown). This confirms the identity of the recombinant protein with homospermidine synthase. Plant homospermidine synthase is essentially dependent on spermidine which functions as donor of the aminobutyl unit, which is transferred onto putrescine (Ober and Hartmann, 1999b; Hartmann and Ober, 2000). Thus, the plant enzyme is not able to synthesize homospermidine from two molecules of putrescine, as is has been demonstrated for the genetically unrelated bacterial homospermidine synthase (Tholl et al., 1996). As expected, the recombinant enzyme from *S. vulgaris* is also strictly dependent on spermidine (Table 1). The low activity in crude extracts in the absence of spermidine is due to contaminating traces of spermidine still present in the desalted extracts.

2.4. Inconsistence of recently published data for HSS of S. vulgaris

The cDNA sequence determined for HSS of *S. vulgaris* roots is not in accordance with a recently published sequence (Kaiser, 1999). The major difference is an additional guanine at position 56 of the already published sequence, resulting in a frame shift upstream of this insertion (Fig. 3). As a consequence the correct start ATG of that sequence is not in the ORF. Instead there is shown a start ATG at position 7, which was found to be the triplett AGG by 5′-RACE technique (Fig. 3). Presumably the submitted sequence does not represent the original sequence of the 5′-terminus. For instance, the *Nde*I-restriction site directly upstream of the ATG initiation codon, which was introduced by a primer, is still present in the sequence.

The ORF determined by this artificially introduced ATG stops after 36 amino acids resulting in a protein of approximately 4.2 kDa.

The specific activity of 10.79 nkat/ mg claimed for crude preparations of the recombinant homospermidine synthase from *S. vulgaris* by Kaiser (1999) is unlikely and by far too high because of two reasons: (a) it was estimated in absence of added spermidine; (b) the highest value determined under optimal conditions for the homogenously purified recombinant enzyme from *S. vernalis* was found to be 3.2 nkat/mg (Ober and Hartmann, 1999b).

3. Experimental procedures

3.1. RNA isolation and cDNA synthesis

Total RNA was extracted from five day old root cultures of *S. vulgaris* using the RNeasy Plant Mini Kit (Qiagen). Total RNA (2 μg) was used for reverse transcription by Superscript II (Life Technologies) as described previously (Ober and Hartmann, 1999a).

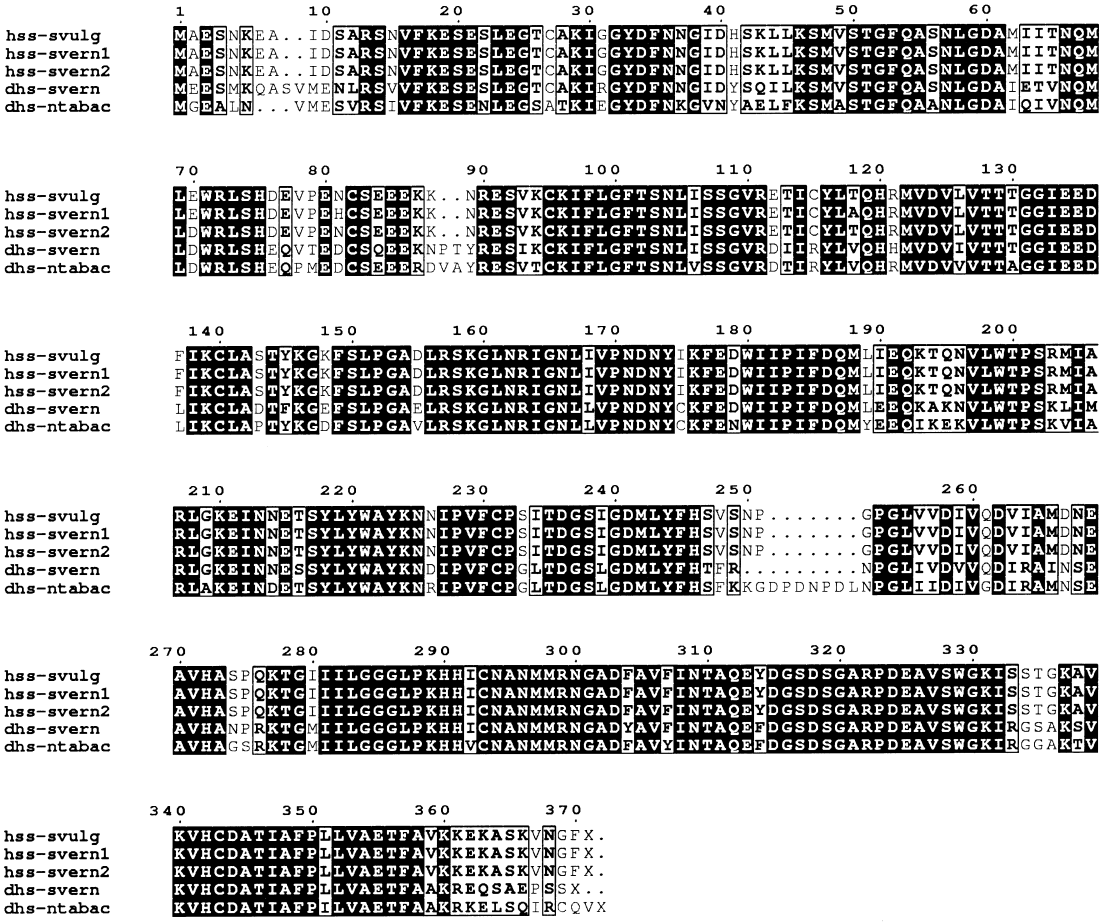

Fig. 1. Alignment of the amino acid sequences of HSS and DHS of different plant species. hss-svulg: HSS of *S. vulgaris*; hss-svern1: HSS of *S. vernalis* sequence 1; hss-svern2: HSS of *S. vernalis* sequence 2; dhs-svern: DHS of *S. vernalis*; dhs-ntabac: DHS of *N. tabacum*.

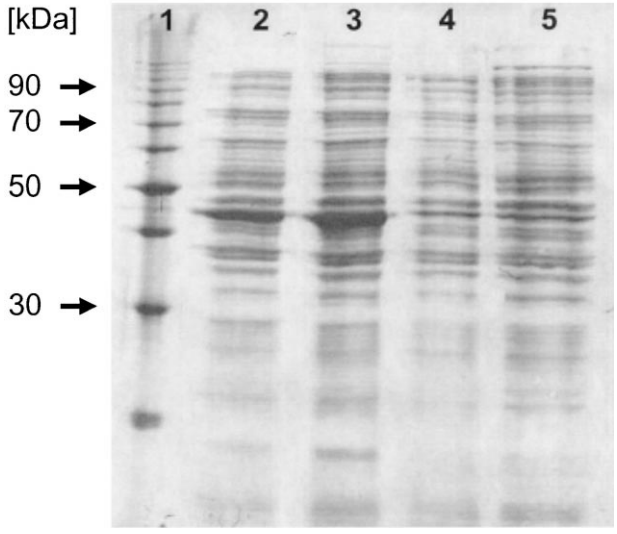

Fig. 2. Expression of recombinant homospermidine synthase of *S. vulgaris* roots. Coomassie stain of a SDS-PAGE on a 12% gel blotted to a PVDF-membrane; 10 kDa protein standard (Life Technologies, lane 1, *E. coli* BL21(DE3) transformed with pET3a/hss-plasmid without induction (lane 2) and after induction with 1 mM IPTG (lane 3), *E. coli* BL21(DE3) untransformed control strain without induction (lane 4) and after induction with 1 mM IPTG (lane 5).

Table 1
Specific activity of recombinant homospermidine synthase from root cultures of *S. vulgaris*. Enzyme activity was determined in crude and dialysed extracts in the presence or absence of spermidine (aminobutyl donor) as indicated, 400 μM putrescine (aminobutyl acceptor) and 1 mM NAD^+

	Crude extract		Desalted extract	
Spermidine	Without	400 μM	Without	400 μM
Specific activity (pkat/mg)	14.1	188.0	nd[a]	241.1

[a] nd: not detectable.

3.2. PCR amplification of full-length homospermidine synthase cDNA from S. vulgaris

To identify the cDNA encoding homospermidine synthase in *S. vulgaris* we applied 3′- and 5′-RACE techniques with the gene specific primers originally designed for the cloning of homospermidine synthase from *S. vernalis* (Ober and Hartmann, 1999b). Primer P1 (5′-dCGA GAC TTC ATA TCT GTA TTG GGC ATA TAA-3′) and an oligo-$(dT)_{17}$ primer (5′-dGTC

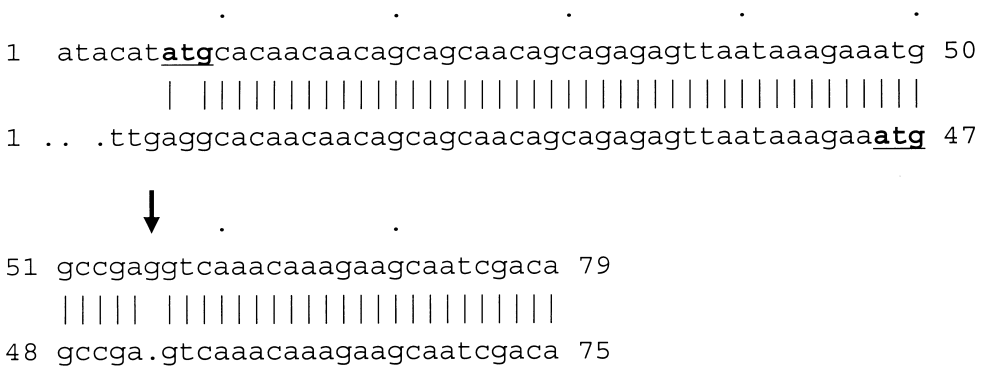

Fig. 3. Alignment of the 5′-end of the nucleic acid sequence of HSS of *S. vulgaris* as it was obtained by 5′-RACE technique (bottom) and as it was published by Kaiser (1999) (top). The start-ATG of both sequences are underlined.

GAC TCG AGA ATT $C(T)_{17}$-3′) were used (each 0.4 μM) to amplify the 3′-end of the cDNA. The 5′-end of the cDNA was amplified using the 5′-RACE System (Life Technologies) according to the manufacturer's instructions and with the primers P2 (5′-dCCC TTT CGA TCT CAA AT-3′) and P3 (5′-dTCT CAA ATC AGC TCC GGG TAA AGA A-3′). The resulting PCR fragments of 685 bp (3′-RACE) and 535 bp (5′-RACE) were purified by electrophoresis and subcloned using the TA Cloning Kit (Invitrogen). For amplification, subcloning and expression of the full-length cDNA two gene specific primers were designed, primer P4 (5′-dTA TAT CAT **ATG** GCC GAG TCA AAC AAA GAA G-3′) and primer P5 (5′-dTAA GGA TCC TTA AAA ACC ATT GAC TTT AGA TGG-3′) containing *Nde*I and *Bam*HI restriction sites (underlined), respectively. After amplification with *Pfu* DNA polymerase (Promega) the resulting fragment of 1130 bp was digested with *Nde*I/*Bam*HI, ligated into a *Nde*I/*Bam*HI-linearized pET-3a expression vector (Novagen), and transformed into *E. coli* XL1-blue cells (Stratagene). Positive transformants containing the pET3a/hss-plasmid were selected by PCR amplification using the primers P4 and P5. The plasmid DNA was purified from one positive clone and subsequently sequenced and transformed for expression in *E. coli* BL21(DE3) (Stratagene). Sequencing was performed using the fluorescence dye terminator technology on ABI Prism sequencers (SeqLab, Göttingen, Germany).

3.3. Expression of *S. vulgaris* homospermidine synthase in *E. coli*

E. coli BL21(DE3) cells containing the pET3a/hss-plasmid were grown in LB-medium containing 50 μg/ml ampicillin for approximately 14 h at 25°C, were induced with 1 mM isopropyl-β-D-thiogalactosid (IPTG) for further 9 h and harvested by centrifugation. The cells were resuspended in standard buffer (0.1 M Glycine/NaOH, pH 9.5, 1 mM DTT, 0.1 mM EDTA), broken by sonification and the supernatant was desalted via PD-10 Desalting Columns (Pharmacia) resulting in a crude extract. To verify the dependency of the HSS-assay on spermidine, the crude extract was dialysed and concentrated by ultrafiltration (Amicon-cell 10 ml, PM30 membrane).

3.4. HSS and DHS assays

HSS- and DHS-activity was determined as described previously (Ober and Hartmann, 1999b).

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