

An *Arabidopsis* cDNA encoding a bifunctional glutamine amidotransferase/cyclase suppresses the histidine auxotrophy of a *Saccharomyces cerevisiae* *his7* mutant

Ko Fujimori^a, Daisaku Ohta^{b,*}

^aTakarazuka Research Institute, Novartis Pharma K.K., 10-66 Miyuki-cho, Takarazuka 665-8666, Japan

^bResearch Institute for Biological Sciences, Okayama 7549-1 Yoshikawa, Kayo-cho, Okayama 716-1241, Japan

Received 26 March 1998; revised version received 27 April 1998

Abstract A cDNA encoding a glutamine amidotransferase and cyclase catalyzing the fifth and sixth steps of the histidine (His) biosynthetic pathway has been isolated from *Arabidopsis thaliana*. The N- and C-terminal domains of the primary structure deduced from a full-length *Arabidopsis* *hisHF* (*At-HF*) cDNA showed significant homology to the glutamine amidotransferase and cyclase of microorganisms, respectively. Effective suppression of the His auxotrophy of a *Saccharomyces cerevisiae* *his7* mutant with the *At-HF* cDNA confirmed that the *At-HF* protein has bifunctional glutamine amidotransferase (HisH) and cyclase (HisF) activities.

© 1998 Federation of European Biochemical Societies.

Key words: cDNA; Glutamine amidotransferase/cyclase; Histidine biosynthesis; *Arabidopsis thaliana*; *Saccharomyces cerevisiae*

1. Introduction

The biosynthesis of histidine (His) in *Escherichia coli* and *Salmonella typhimurium* has been extensively characterized biochemically and genetically [1]. The complete nucleotide sequences of the genes involved in the His biosynthetic pathway have been determined [2], and it was shown that the 11 enzymatic activities are encoded by eight genes organized in a single operon [2]. Recently, His biosynthetic genes have also been isolated from a variety of organisms including lower eukaryotes such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Neurospora crassa* [3]. From higher plants, the cDNAs encoding the histidinol dehydrogenase (HDH) of *Brassica oleracea* [4] and the imidazoleglycerolphosphate dehydratases (IGPD) of *Arabidopsis thaliana* and *Triticum aestivum* [5,6] have been isolated.

In most eubacteria, the His genes are organized in an operon as described above, while those in the lower eukaryotes are scattered throughout the chromosomes [7]. In archaeobacteria such as *Methanococcus vanielii* and *M. jannaschii*, the His genes are not organized as an operon [8,9]. Not only the overall gene organization but the structures of each gene are variable among different organisms. For example, in *S. cerevisiae*, *HIS4* and *HIS7* encode multifunctional enzymes [10,11]. The *HIS7* gene of *S. cerevisiae* encodes the bifunctional glutamine amidotransferase (HisH) and the cyclase (HisF), although both proteins in eubacteria are encoded by independent cistrons, *hisH* and *hisF*, which are interrupted by the *hisA* cistron

[2,12]. In higher plants, the HDH protein catalyzes the tenth and eleventh steps of the His pathway [4], as does the HisD protein in eubacteria [2], while the IGPD in higher plants is not accompanied by a histidinolphosphate phosphatase domain [5], both of which are encoded by *hisB* in most eubacteria [2,12]. On the other hand, phosphoribosyl (PR)-AMP cyclohydrolase (PRA-CH) and PR-ATP pyrophosphohydrolase (PRA-PH), encoded by a single gene, *hisIE* in eubacteria [2], were encoded by independent genes in *M. vanielii* and *M. jannaschii* [8,9] and *Azospirillum brasilense* [13].

In this paper, we report the isolation and characterization of an *Arabidopsis* cDNA encoding a bifunctional glutamine amidotransferase/cyclase. Functional expression of the *Arabidopsis* *At-HF* cDNA in a *S. cerevisiae* *his7* defective mutant demonstrated that the *At-HF* cDNA encodes a single polypeptide having bifunctional activities for glutamine amidotransferase (HisH)/cyclase (HisF).

2. Materials and methods

2.1. Plant material, growth and microbial strains

A. thaliana ecotype Columbia (Col-0) (Lehle Seeds, Tucson, AZ, USA) were grown under sterile conditions on 0.8% (w/v) agar plates containing GM medium [14] in a growth chamber maintained at 23°C and 80% relative humidity with a 16-h light/8-h dark cycle. *S. cerevisiae* strain SH782 (*MATa ura3-52 leu2-3,112*) was a generous gift from S. Harashima (Osaka University, Suita, Japan) for the construction of the *HIS7* disruption mutant. *E. coli* strain DH5α was used as a host for the propagation and manipulation of plasmid DNAs. The media for yeast were as described [15].

2.2. Isolation of an *Arabidopsis* cDNA encoding a bifunctional glutamine amidotransferase/cyclase

An *Arabidopsis* expression sequence tag (EST) clone (186B18T7; GenBank accession number H37732) was identified as a putative cyclase (HisF) of *Arabidopsis* through the BLAST search against the *Arabidopsis* ESTs [16,17] with the aid of the primary structure deduced from the *S. cerevisiae* *HIS7* gene [11]. In order to isolate full-length cDNAs corresponding to the EST clone, total RNA was isolated from 7-day-old *Arabidopsis* seedlings by the acid guanidinium thiocyanate-phenol-chloroform extraction method [18], and a DNA fragment corresponding to the EST clone was amplified employing a reverse transcription (RT)-polymerase chain reaction (PCR) strategy. Briefly, first strand cDNA corresponding to the EST clone was synthesized using a specific antisense primer PR160 (5'-CCAACTGCTTGCGATCACCGG-3') and Superscript II RNase H⁻ Reverse Transcriptase (Gibco-BRL, Rockville, MD, USA). Prior to the RT reaction, the total RNA (5 µg) was heat-denatured at 72°C for 3 min, and the first strand cDNA synthesis was performed at 42°C for 60 min followed by heat inactivation at 95°C for 3 min. PCR was performed using a set of specific primers of PR159 (5'-GAAATCAGGCAGTGGTTGTAAG-3') and PR160, ExTaq DNA Polymerase (Takara Shuzo, Kyoto, Japan) and the RT reaction products [19]. After the initial denaturation at 95°C for 5 min, PCR was carried out with 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 30 cycles.

*Corresponding author. Fax: (81) (866) 56-9454.

E-mail: ohtad@mth.biglobe.ne.jp

A PCR-amplified 320-bp DNA fragment was directly cloned into a pCR2.1 vector (Invitrogen, San Diego, CA, USA), yielding plasmid pKF405. The nucleotide sequence of the insert DNA of pKF405 was confirmed to contain the expected DNA fragment (data not shown).

An *Arabidopsis* cDNA library (7-day-old) [20] constructed with a λ ZAPII vector system (Stratagene, La Jolla, CA, USA) was screened with the 32 P-labeled cDNA insert of pKF405. Prehybridization, hybridization and washing were carried out as described [21].

The nucleotide sequences were determined on both strands by the dideoxynucleotide chain termination method [22] using an ABI PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).

2.3. Construction of the *S. cerevisiae* *his7* mutant

A *HIS7* disruption mutant of *S. cerevisiae* BY1006 was constructed as follows. A region encoding the *HIS7* ORF of *S. cerevisiae* was amplified by PCR using PRI175 (5'-GCAAGCTTATGCCGGTCTGTT-CACGTGATTGAC-3') and PRI176 (5'-GCCTCGAGCCACATTA-

CTCTTCATCCATTC-3') as primers and chromosomal DNA of *S. cerevisiae* strain S288C (*MATa mal mel gal2*) as the template. PCR products double-digested with *Hind*III and *Xho*I were cloned into a pYES2 vector (Invitrogen) to yield plasmid pKF416. The 1.0-kb *Sall*-*Sall* fragment from pKF416 was replaced with the 2.2-kb *Sall*-*Xho*I fragment of *LEU2* gene [23] to obtain plasmid pKF426. *S. cerevisiae* SH782 was transformed with a 2.9-kb *Hind*III-*Xho*I fragment from pKF426 [24] and incubated on synthetic complete (SC) plates supplemented with 2% (w/v) glucose (Glu) and an amino acids mixture without leucine (Leu) (SC+Glu-Leu) at 30°C for 3 days. The selection for Leu⁺ phenotype in the presence of L-His resulted in strain BY1006 (*MATa ura3-52 leu2-3,112 his7::LEU2*).

2.4. Expression in *S. cerevisiae* *his7* defective mutant

The following regions were amplified by PCR using sets of gene specific primers: a 1900-bp coding region (nucleotide positions +1 to +1900, taking the A of the first ATG codon as +1) using PRI102 (5'-CGGAATTCATGGAGGCTACGGCGGCGCC-3') and PRI103

		PRI102		
-5	TATCAATGGAGGCTACGGCGGCGCCATTCTCTCTCAATTGTCTCTCCAGACAAAACCTCTCTTCATCTCTTCGATTCGC			25
	M E A T A A P F S S I V S S R Q N F S S S S S I R			
75	GCTTCTCTCCGGCTTCTTTATTCCTCTCCAGAAGAGTATTGGCAATGTTAATCGCAAATCAAACTCTCCAGAAGCCT			52
	A S S P A S L F L S Q K S I G N V N R K F K S P R S L			
155	CTCCGTCGCGCATCTTCTACCTCAGATTCTGTTGTGACTTTGCTTGACTACGGAGCTGGAATGTTCCGGAGCATCCGCA	PRI122		78
	S V R A S S T S D S V T L L D Y G A G N V R S I R			
235	ATGCTCTTCGTCATCTCGGCTTCAGCATCAAAGACGTTCAAACGCGGGAGACATTCTGAATGCTGACTCATATTT			105
	N A L R H L G F S I K D V Q T P G D I L N A D R L I F			
315	CCAGCGTGTGGCCTTTTGCACCGCCATGGATGACTTAACAGAACTGGGATGGCTGAAGCTTTGTGCAATATATTGA			132
	P G V G P F A P A M D V L N R T G M A E A L C K Y I E			
395	GAATGACCGTCCATTTCTAGGCATATGCTTGGTCTACAACACTTTTCGATTCTAGTGAACAGAATGGACAGTCAAG			158
	N D R P F L G I C L G L Q L L F D S S E Q N G P V K			
475	GTCTTGTGTGATACCGGAATAGTTGGACGCTTTGATGCTTCAGCTGGTATAAGAGTACCCACATTGGCTGGAATGCT			185
	G L G V I P G I V G R F D A S A G I R V P H I G W N A			
555	TTGCAAGTTGGGAAGGATTCTGAATTTTGGATGATGTTGGAACCGTCATGCTATTTTGTTCATTCGTACAGGGCCAT			212
	L Q V G K D S E I L D D V G N R H V Y F V H S Y A I			
635	TCCATCAGATGAAATAAGGACTGGATTTCTGCTACCTGTAATTATGGTGAATCATTATATCTTCCATAAGAAGGGGAA			238
	P S D E N K D W I S S T C N Y G E S F I S S I R R G			
715	ATGTGATGCAGTTCAATTCATCTGAAAGAGCGGGGAGGTGGGCTTTCTGTTTAAAGAGGTCTTGCATCAAAA			265
	N V H A V Q F H P E K S G E V G L S V L R R F L H P K			
795	TTACCTGCAACACAGAAGCCAATGGAAGAAAGGCTCAAACTTGCAAGAGGGTATTGCTTGTCTGTATGTGAGGAC			292
	L P A T Q K P M E G K A S K L A K R V I A C L D V R T			
875	GAATGATAAAGGAGATCTCGTAGTTACTAAAGGGGATCAGTATGATGTGAGAGCAATCTAATGAAACGAGGTTGAA			318
	N D K G D L V V T K G D Q Y D V R E Q S N E N E V			
955	ACCTTGGCAAACCTGTTGATTGGCTGGGCGATATTACAAAGATGGTGCAGATGAGATTAGCTTTTAAACATACTGGA			345
	N L G K P V D L A G Q Y Y K D G A D E I S F L N I T G			
1035	TTCCGCGATTTTCTCTAGGGGATTTGCCGATGATTCAGGTGTTGAGGCGAGACATCAAGAAATGCTTTGTACCACTAAC			372
	F R D F P L G D L P M I Q V L R Q T S K N V F V P L T			
1115	TGTTGGAGGTGTTATAGAGACTTTACAGATGCTAGTGGCAGGTACTATTCTAGCTTGAAGTTGCTGCTGAGTATTCA			398
	V G G G I R D F T D A S G R Y Y S S L E V A A E Y F			
1195	GATCCGGTGTGATAAGATGTCATAGGAAGTGACGCTGTTTGTGTCGAGGAGTTCATAAAATCAGGGGTGAAGACA			425
	R S G A D K M S I G S D A V F A A E E F I K S G V K T			
1275	GGAAAGAGTAGTTAGAACAGATATCCAGAGTTTATGGAATCAGGAGTGGTGTAAATGATTGATCCTCGTAGAGTTTA	PRI59		452
	K S S L E Q I S R V Y G N Q A V V V S I D P R V Y			
1355	TGTGAACCATCCGGATGATGTGCCATACAAAGTCATCAGAGTAACCTAATCCAGGCCAAATGGAGAAGAATATGCCTGGT			478
	V N H P D D V P Y K V I R V T N P G P N G E E Y A W			
1435	ATCAGTGCACGGTTCAGTGGAGGACAAGAAGTCGACCTATTGGAGCATTTGAGCTTGGCAAAGCGGTTGAAGAATTAGGT			505
	Y Q C T V S G G Q E G R P I G A F E L A K A V E E L G			
1515	GCCGGTGAATACTATTGAACTGCATAAAGTGTGATGGTCAAGGAAAGGATTCGACATAGACTTAGTAAAGCTCATCTC			532
	A G E I L L N C I N C D G Q G K G F D I D L V K L I S			
1595	AGATTTCAGTAGGCATACCGGTGATCGAAGCAGTGGAGCAGGTACTCCCGACCACTTTTCCGAGGTGTTGAAGAAGACA	PRI60		558
	D S V G I P V I A S S G A G T P D H F S E V F E D			
1675	AACGCATCTGCCGCTTGTGTCGGCATTTTCCACCGAAAGAGGTACCAATCCCAATCTGTGAAAGAGCACTTACAA			585
	K R I C R A C C R H F P P E R G Y Q S Q S V K E H L Q			
1755	GAGGAGCGCATAGAAGTCAGGATCTGAAATTTCTGGTCTGCGTGTACCATATTCATGACACATTCAACAAGATAGA			593
	E E R I E V R I *			
1835	ATCTTTACCTTCAATAAATAAATTAAAGTCGAACCATCGGAGCTCAATAGGTCTGAGTGAATCCTCTTATAGTTGATT	PRI103		
1915	TCCTTTTGTGACAGTAAGACTGATACAAATTTATGGATTTCCCTTGAAAGTAAGAAAGCAACGTATGTTCACTTAAAAA			
1995	AAAAAAAAAA			

Fig. 1. Nucleotide and predicted amino acid sequences of the *Arabidopsis* *At-HF* cDNA. Numbers shown on the left and right of the columns refer to nucleotide and amino acid sequences, respectively. Asterisk indicates the stop codon. Primers used are indicated by arrows. Possible polyadenylation signal is underlined. The DDBJ accession number of the *Arabidopsis* *At-HF* cDNA is AB006210.

(5'-CGCTCGAGGATTCACTCAGACCTATTGG-3'), a 1717-bp coding region truncated in its putative chloroplast transit peptide (nucleotide positions +184 to +1900) using PRI122 (5'-CGGAATC-CATGGTTGTGACTTGTGCTGACTAC-3') and PRI103. Each of those PCR products digested with *Eco*RI and *Xho*I was cloned into a pYES2 vector to obtain plasmids pKF433 and pKF434, respectively. Each of those plasmids was used for the transformation of *S. cerevisiae* BY1006 (*his7*). The transformants were selected by uracil auxotrophy and incubated on SC plates containing 2% (w/v) galactose (Gal) and appropriate amino acids (SC+Gal-Ura-Leu) for 3 days at 30°C. The pKF416 carrying the DNA insert containing the open reading frame (ORF) of *S. cerevisiae* HIS7 protein was used for the transformation of strain BY1006 as a positive control. The *S. cerevisiae* transformants were analyzed for their ability to grow on minimal galactose plates without L-His (SC+Gal-Leu-His).

2.5. Southern and Northern blot hybridization analyses

Genomic Southern and Northern blot analyses were carried out as described by Sambrook et al. [21].

3. Results and discussion

3.1. Isolation of an *Arabidopsis* cDNA encoding bifunctional glutamine amidotransferase and cyclase

Recent genetic studies revealed the substantial conservation of the protein primary structures of the His biosynthetic enzymes of prokaryotic and lower eukaryotic origins [3]. This sequence conservation has also been verified with respect to the His biosynthetic pathway enzymes of higher plants [4,5].

At-HF	1	MEATAAPFSSIVSSRQNFSSSSSIRASSPASLFLSQSIGNVNRKFKSPRSLSVRASSTSDSVITLLDYG
Sc HIS7	1	MPVNHVIDVE
Ec hisH	1	MNVVILDIG
Sy hisH	1	MGYIAVDYD
Mj hisH	1	MMIGIIDYN
At-HF	71	ACNVRISIRNALRHILGFSIKDVQTPGD...ITLADRLIEPGVGFAPAMDVL...NRTQMAEALCKYITENDRE
Sc HIS7	11	SCNLQSLTINALEHLYGEVQLVKSPKDFNTSGTSRLILPGVGNVYAHFVDNL...FNRGFEKPIREYTESGKF
Ec hisH	10	CANLNSVKSLIARHGYPEKVSRLDPDV...VLADKLELPGVGTQAAMQV...REBELFDLIK...ACTOF
Sy hisH	11	MCNLESVCKGLEKVGNNPLVTDQAHV...HDGATAIVLPGVGSFDPVQHIL...RARGLEEVLTAKGIF
Mj hisH	11	AGNLESIQKE...VELYDKVIITNNSSE...LACIKIILPGVGNFGSAMENLAPLKETIYKIVDDR...VF
At-HF	205	FLGICLGLDLEFDSSEONGPVKGLGVHPCIVGREDASACIRVPHIGNALQVQKDSSEIT...DDVGNRHVM
Sc HIS7	145	INGICVGLDLEFAGSVESPKSTGLNLYIDFKLSRFDDSEK...PVPEIGINS...CIPSENLF...GLDPYKRYF
Ec hisH	141	VLGICLGMOLIGRRSEESNGVDLGLITDELVPKMT...DFGLPLPHMGNNRVYPOAGNR...LFOGIEDGAYFY
Sy hisH	146	FLGICLGLDLEFDSSEEGQET...GLGITEGVKHFRESEPLTI...PHMGANGLQFNQPDCLWQDLPEPEQVY
Mj hisH	142	FLGICLGMOLIFEESEKRGKGLGITKGNVIRK...DVE...KLPHMGNSVKIVKDCP...LPEIKNNSYFY
At-HF	201	FVHSYRAIESDENK...DWISSTCNYG...ESFISIRRRGNHBAVQFHPKSGEVLGSLVRRFLH...
Sc HIS7	141	FVHSFAAILNSEKKKNLENDGWKIAKAKYGESEFIDAAVNNNIFATOFHPKSGKAGLVNIENFLKQOSF
Ec hisH	141	FVHSYAM...PVNFWT...IAQ...CNYGEP...FTAAVQKDNFYGVQFHPEKPSAAGARLIRNFLEM*
Sy hisH	141	FVHSYMAFLDPQV...IAASTTHSQTIAAATAKDNMAVOFHPEKSESTLGLKILANFVKKVAL
Mj hisH	141	FVHSYHVNPEDEDCI...VGK...TEYGR...EPPSVINKDNVFAOFHPKESGKILGLIENFVELL*
At-HF	264	...PKLPATQKPM...EGKASKLAKRVIACLDVEITNDKGLDVVITKGD...YDVREQSNENEVNLGHPVILAG
Sc HIS7	215	PIPNYSABEEKELLMNDYSNYG...ITRIITACLDVEITNDQGDVVTIKGD...YDVREKSDGKGVNLRHPVQLAQ
Ec hisH		
Sy hisH	208	SDI*
Mj hisH		
Ec hisF	1	MLAKRIIPCLDVEDGO...VVKGVDF...RNHEITIGITVELAK
Sy hisF	1	MTLAKRIIPCLDVNAGR...VVKGINF...VDLQDAGDPVELAR
Mj hisF	1	MTLAKRIIPCLDIKDR...VVKGTF...LNLFDAGDPVELAQ
At-HF	329	QNYKDGADLEISFLNITGFRDFPLGLDLPMTJOLVLRQISKNVFLVPLTVGGGIRDFDASGRYSSLEVAEYF
Sc HIS7	285	KNYQQGADLVIFLNTISFRDCLPMDLPMLEVLKQAAKIVFVPLTVGGGIDVDVDTGKIPALEVASLYF
Ec hisF	38	RKAEEGADLVFYDITASSE...GRVVDKSWSRVAEVIDIHFVAGGIRSLDAA...KIL
Sy hisF	39	AKNEAGADLVFLDITATHE...QDITI...DWVYRTAEVFIPLTVGGGISTLPHIK...NLJ
Mj hisF	38	YMDDEGADLVFLDITASAE...KEDII...DWVYRTAEVFIPLTVGGGISTLPHIK...RIL
At-HF	399	RSGADKMSIGDAVFAAEFIKSGVKT...GKSSLEQISRVYENLAWVSIDPREVYVNHDPDDVPYKIRVT
Sc HIS7	255	RSGADKVSIGDAVYAAEKYYELGNRGDGTSP...ETTSKAYGACAVVTSVDPKRVVYVNSQADTKNKFETE
Ec hisF	94	SFGADKHSINSEFAL...ADFTLITRLADRFVQGITVVGIDTWYDAETGKYHNYQT...
Sy hisF	95	RAGADKVSINSSAV...RDGDFISRASDRFGRCIVVAIDARIRLDADNPG...
Mj hisF	94	RAGADKVSINTAV...KNENLKEASEIFGSCVVAIDAKHVNNEDEIDKINKNVVK
At-HF	468	NPGPNGEYAYQCTVSGGQGRPIGAFELAKAVEBLGAGEILLNCLDQGGKSEFIDVVLISDSVGI
Sc HIS7	425	YPGPNGEYAYQCTIKGGRESDLGWELTRACBALGAGEILLNCLDQGSNSGYDLLEHVKDAVKI
Ec hisF	147	...GDESRTVITQWETLDVQVBYQKRGAGETV...LNMNDGVVNSGYDLLEHVKVREVCV
Sy hisF	143	...DVYVRGRENGLD...IAAFAEYAKRGAGELVITSMFGDQTA...YDLALTAFAERVEI
Mj hisF	151	...VEDGTCFVYIYGGKRETDG...INFAKVEBLGAGEILLNCLDQGTGSYDLLEHVKVREVCV
At-HF	538	PVIASSGAGTIDHFSVFEEDL...RICRACCRHPPPERGYQSQSV...EHLQEERIEVRI*
Sc HIS7	495	PVIASSGAGVPEHFEAFILKTRADACLGGM...EHRG...EFTVNDVVEYLLHGLKVRMDEE*
Ec hisF	203	PVIASSGAGIMEHLEAFRDADVGCALAASV...FHKQIINTIGELHAYLATQGVETIC*
Sy hisF	202	PVIASSGAGNCQHVYFAFTEGKABEALLASL...DHYQLTITIGELTFLEARQIPVHTAVCG*
Mj hisF	217	PVIASSGAGKPEHVYFAFVYGAADAALMAGI...LHYREYTIETIKTYCADRGIFMILL*

Fig. 2. Alignment of the predicted amino acid sequences of *Arabidopsis* At-HF, *S. cerevisiae* HIS7 (accession number X69815) [11] and *hisH*, *hisF* gene products from *E. coli* (accession number X13462) [2], *Synechocystis* subsp. PCC6803 (accession numbers D64004, D90912) [25] and *M. jannaschii* (accession numbers U67493, U67500) [9]. Conserved amino acid residues are shaded.

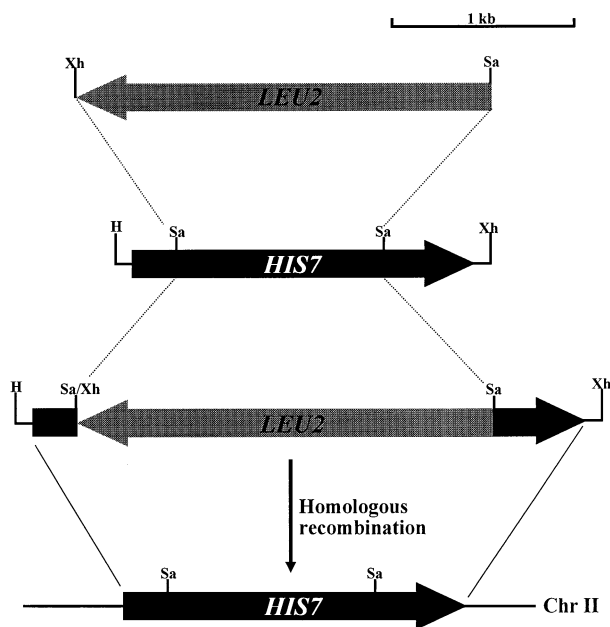


Fig. 3. Construction of the *S. cerevisiae* *his7::LEU2* null allele, BY1006. Restriction enzyme sites: H, *Hind*III; Sa, *Sal*I; Xh, *Xho*I.

In this study, we report a novel cDNA encoding a glutamine amidotransferase/cyclase, the *At-HF* from *A. thaliana*. A database search against the ESTs using the *HIS7* protein sequence hit an EST clone (186B18T7) of *A. thaliana*. The amino acid sequence encoded by this clone showed a high degree of similarity to the C-terminal portion of the bifunctional glutamine amidotransferase/cyclase (*HIS7*) from *S. cerevisiae*. An *Arabidopsis* cDNA library was then screened for a full-length cyclase cDNA using a PCR-amplified 186B18T7 sequence as a probe, and four positive clones out of 50 000 plaques were finally isolated. From the partial DNA sequencing, these four clones were identical except for different lengths at their 5' ends (data not shown). The plasmid harboring the longest insert was designated pKF412. The cDNA insert of pKF412 is 2011 bp long and contains an ORF encoding a polypeptide of 593 amino acids with a molecular mass of 64 720 Da (Fig. 1). Analysis of the 5'-untranslated region of the *Arabidopsis At-HF* gene revealed an in-frame stop codon (TAG) at 84 nucleotides upstream of the putative translation initiation codon (ATG) of the pKF412 insert (Fujimori et al., unpublished data), indicating that the ORF found in the pKF412 encodes a full-length transcript.

3.2. Amino acid sequence comparison

The amino acid sequence deduced from the *At-HF* cDNA was compared to those of microorganisms currently available on the GenBank/EMBL/DDBJ/SwissProt databases (Fig. 2). Sequence alignment indicated that the N-terminal domain encompassing Val-64 to Leu-262 and the C-terminal domain from Leu-280 to Phe-555 of the *At-HF* protein showed significant homology to the glutamine amidotransferase (*HisH*) and the cyclase (*HisF*) from microorganisms, respectively (Fig. 2). In yeast *S. cerevisiae*, the glutamine amidotransferase (*HisH*) and the cyclase (*HisF*) domains are located at the N-terminal half and C-terminal half of a single polypeptide encoded by *HIS7*, respectively [11].

Current results indicated that the amidotransferase/cyclase

activities resided in a single polypeptide encoded by a single gene in *A. thaliana* like that found in *S. cerevisiae*. In contrast, in enterobacteria such as *E. coli* and *S. typhimurium*, the proteins are encoded by two independent cistrons in the same operon, *hisH* and *hisF* [2]. On the other hand, in archaeobacteria such as *M. vanielii* and *M. jamaeschii* [8,9], and *Synechocystis* subsp. PCC6803 [25], the two genes are scattered throughout the chromosome. These observations imply different evolutionary processes or unique chromosomal gene organization processes of the glutamine amidotransferase/cyclase genes in these organisms, but the high sequence similarity and catalytic domains have been conserved (Fig. 2).

Sequence comparison (Fig. 2) revealed an N-terminal extension of approximately 60 amino acids of the *At-HF* protein exhibiting properties typical of chloroplast transit peptides, being rich in hydroxylated residues and a few negatively charged residues [26]. This is consistent with the previous findings that the proteins of the IGPD of *T. aestivum* [6] and the HDH of *B. oleracea* [27] were immunochemically detected in intact chloroplast fractions.

3.3. Functional complementation of a *S. cerevisiae* *his7* mutant with the *At-HF* cDNA of *Arabidopsis*

In order to investigate whether the *Arabidopsis* cDNA cloned in pKF412 actually encodes a bifunctional glutamine amidotransferase (*HisH*) and cyclase (*HisF*), we employed complementation analysis using a *S. cerevisiae* *his7* mutant, of which the DNA region of *HIS7* ORF has been replaced with *S. cerevisiae* *LEU2* gene (Fig. 3). This *His* auxotrophic mutant of *S. cerevisiae* (BY1006) is defective in both enzymatic activities of the glutamine amidotransferase and cyclase, and is thus no longer able to grow on minimal plate without L-His (SC+Glu–Leu–His) (Fig. 4). Mutant strain BY1006 (*his7*) was transformed with either pKF416 (containing the *S. cerevisiae* *HIS7* ORF), pKF433 (bearing the *At-HF* ORF), pKF434 (harboring the *At-HF* ORF truncated in its putative chloroplast transit peptide portion) or pYES2, and was cultivated on SC+Glu–Ura–Leu plates for 3 days at 30°C. Strain BY1006 carrying either pKF433 or pKF434 was able to grow on the SC+Gal–Leu–His plates at the same level as that transformed with pKF416 (Fig. 4). These results indicated that the *At-HF* cDNA cloned in pKF412 encodes a functional glutamine amidotransferase/cyclase of *Arabidopsis*. Since there was no significant difference in the growth between BY1006/pKF433 and BY1006/pKF434, it can be concluded that the N-terminal extension of the *At-*

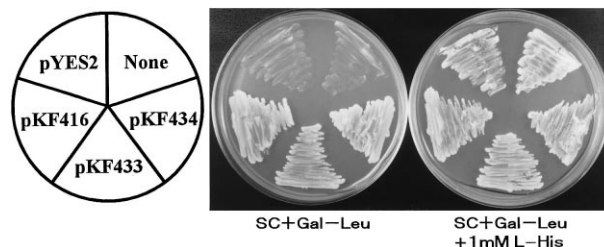


Fig. 4. Suppression of the *His* auxotrophy in *S. cerevisiae* *his7* mutant BY1006. Strain BY1006 was transformed with either pKF416 (containing the *S. cerevisiae* *HIS7* ORF), pKF433 (bearing the *A. thaliana At-HF* ORF), pKF434 (harboring the *A. thaliana At-HF* ORF truncated in its putative chloroplast transit peptide region) or pYES2 and cultivated on SC+Gal–Leu plate in the presence or absence of 1 mM L-His.

HF protein was not required for the enzymatic activities for glutamine amidotransferase/cyclase (Fig. 4). It is therefore possible that this N-terminal portion was in fact a chloroplast transit peptide, as is found with other His biosynthetic enzymes of higher plants [4,5]. Further biochemical studies are needed to confirm the chloroplastic localization of the At-HF protein.

3.4. Southern blot analysis

Fig. 5 shows a genomic Southern blot analysis for the *At-HF* gene of *Arabidopsis*. *Arabidopsis* genomic DNA was digested with the enzymes described below and hybridized with the α - 32 P-labeled full-length *At-HF* cDNA. Digestion with the enzymes *Sma*I or *Xho*I (no restriction sites in the *At-HF* cDNA) gave a single hybridization signal, whereas digestion with *Bgl*II, *Eco*RV or *Hind*III (each of which contains a single restriction site in the cDNA) gave rise to two or more hybridization bands (Fig. 5). No more additional signals were observed even after long exposure (data not shown). These results indicated that there was a single copy of the *At-HF* gene in the *Arabidopsis* genome.

3.5. Expression of the *At-HF* gene in *Arabidopsis*

To study the expression patterns of the *At-HF* gene, Northern blot analysis was performed with total RNA samples from various tissues using an α - 32 P]dCTP-labeled full length *At-HF* cDNA as a probe. The size of the hybridization signal of approximately 2.0 kb corresponds well to the transcript size predicted from the cDNA size (Fig. 6). The *At-HF* gene was expressed similarly in all tissues throughout development. A ubiquitous expression pattern was also noted for IGPD [5]. These results indicated that there are no specific organs/tissues

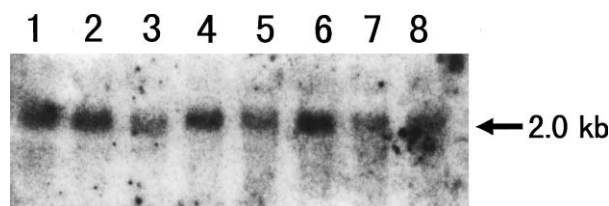


Fig. 6. Northern blot analysis of the *At-HF* gene expression. 10 μ g of total RNA prepared from 1-week-old *Arabidopsis* (lane 1), roots from 2-week-old plants (lane 2), leaves from 2-week-old plants (lane 3), roots from 3-week-old plants (lane 4), leaves from 3-week-old plants (lane 5), roots from 4-week-old plants (lane 6), leaves from 4-week-old plants (lane 7), inflorescence stems from 4-week-old plants (lane 8) was electrophoresed in a 2.2 M formaldehyde-1.2% (w/v) agarose gel, and hybridized with the 32 P-labeled full-length *At-HF* cDNA. Equal loading of RNA was confirmed by staining the gel with ethidium bromide before transfer onto the membrane (data not shown).

that produce His, but rather this amino acid is synthesized and supplied throughout the plant.

At least two plant His biosynthetic enzymes, IGPD [6] and HDH [27], have been shown to be localized at chloroplasts. The His biosynthesis is an energy consuming process, where 41 ATP molecules are utilized for the synthesis of each His molecule [28]. It is therefore advantageous for plants to compartmentalize the entire His pathway in chloroplasts. Intracellular localization of the At-HF together with other His pathway enzymes remains to be clarified through biochemical means.

Acknowledgements: We are grateful to Dr. Satoshi Harashima for providing the *S. cerevisiae* strain SH782, and Nobuko Uodome for her technical assistance.

References

- [1] Winkler, M.E. (1987) Biosynthesis of Histidine, American Society for Microbiology, Washington, DC.
- [2] Carlomagno, M.S., Chiariotti, L., Alifano, P., Giulia, A. and Bruni, C.B. (1988) J. Mol. Biol. 203, 585–606.
- [3] Alifano, P., Fani, R., Liò, P., Lazcano, A., Bazzicalupo, M., Carlomagno, M.S. and Bruni, C.B. (1996) Microbiol. Rev. 60, 44–69.
- [4] Nagai, A., Ward, E., Beck, J., Tada, S., Chang, J.-Y., Scheidegger, A. and Ryals, J. (1991) Proc. Natl. Acad. Sci. USA 88, 4133–4137.
- [5] Tada, S., Volrath, S., Guyer, D., Scheidegger, A., Ryals, J., Ohta, D. and Ward, E. (1994) Plant Physiol. 105, 579–583.
- [6] Tada, S., Hatano, M., Nakayama, Y., Volrath, S., Guyer, D., Ward, E. and Ohta, D. (1995) Plant Physiol. 109, 153–159.
- [7] Mortimer, R.K., Romano, P., Suzzi, G. and Polsinelli, M. (1994) Yeast 10, 1543–1552.
- [8] Beckler, G.S. and Reeve, J.N. (1986) Mol. Gen. Genet. 204, 133–140.
- [9] Bult, C.J., White, O., Olsen, G.J., Zhou, L., Fleischmann, R.D., Sutton, G.G., Blake, J.A., FitzGerald, L.M., Clayton, R.A., Gocayne, J.D., Kerlavage, A.R., Dougherty, B.A., Tomb, J.-F., Adams, M.D., Reich, C.I., Overbeek, R., Kirkness, E.F., Weinstock, K.G., Merrick, J.M., Glodek, A., Scott, J.L., Geoghagen, N.S.M., Weidman, J.F., Fuhrmann, J.L., Nguyen, D., Utterback, T.R., Kelley, J.M., Peterson, J.D., Sadow, P.W., Hanna, M.C., Cotton, M.D., Roberts, K.M., Hurst, M.A., Kaine, B.P., Borodovsky, M., Klenk, H.-P., Fraser, C.M., Smith, H.O., Woese, C.R. and Venter, J.C. (1996) Science 273, 1058–1073.
- [10] Donahue, T.F., Farabaugh, P.J. and Fink, G.R. (1982) Gene 18, 47–59.
- [11] Kuenzler, M., Balmelli, T., Egli, C.M., Paravicini, G. and Braus, G.H. (1993) J. Bacteriol. 175, 5548–5558.

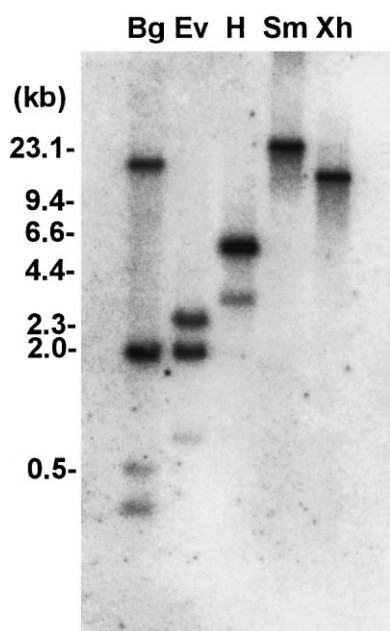


Fig. 5. Genomic Southern blot analysis. Total genomic DNA prepared from 2-week-old grown *Arabidopsis* seedlings was digested with several restriction enzymes as indicated (Bg, *Bgl*II; Ev, *Eco*RV; H, *Hind*III; Sm, *Sma*I; Xh, *Xho*I). 10 μ g of the digested DNA was separated on a 0.7% (w/v) agarose gel and hybridized with the 32 P-labeled full-length *At-HF* cDNA under low stringency condition. Molecular weight markers are shown on the left.

- [12] Fleischmann, R.D., Adams, M.D., White, O., Clayton, R.A., Kirkness, E.F., Kerlavage, A.R., Bult, C.J., Tomb, J.-F., Dougherty, B.A., Merrick, J.M., McKenney, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J.D., Scott, J., Shirley, R., Liu, L.-L., Glodek, A., Kelley, J.M., Weidman, J.F., Phillips, C.A., Spriggs, T., Hedblom, E., Cotton, M.D., Utterback, T.R., Hanna, M.C., Nguyen, D.T., Saudek, D.M., Brandon, R.C., Fine, L.D., Fritchman, J.L., Fuhrmann, J.L., Geoghagen, N.S.M., Gnehm, C.L., McDonald, L.A., Small, K.V., Fraser, C.M., Smith, H.O. and Venter, J.C. (1995) *Science* 269, 496–512.
- [13] Fani, R., Alifano, P., Allotta, G., Bazzicalupo, M., Carlomagno, M.S., Gallori, E., Rivellini, F. and Polsinelli, M. (1993) *Res. Microbiol.* 144, 187–200.
- [14] Valvekens, D., Montagu, M.V. and Lusebettens, M.V. (1988) *Proc. Natl. Acad. Sci. USA* 85, 5536–5540.
- [15] Rose, M.D., Winston, F. and Hieter, P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [16] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) *J. Mol. Biol.* 215, 403–410.
- [17] Newman, T., de Bruijn, F.J., Green, P., Keegstra, K., Kende, H., McIntosh, L., Ohlrogge, J., Raikhel, N., Somerville, S., Thomas, M., Retzel, E. and Somerville, C. (1994) *Plant Physiol.* 106, 1241–1255.
- [18] Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159.
- [19] Saiki, R.K., Gelfand, D.H., Stofeel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) *Science* 239, 487–491.
- [20] Mizutani, M., Ohta, D. and Sato, R. (1997) *Plant Physiol.* 113, 755–763.
- [21] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [22] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- [23] Andreadis, A., Hsu, Y.P., Kohlhaw, G.B. and Schimmel, P. (1982) *Cell* 31, 319–325.
- [24] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) *J. Bacteriol.* 153, 163–168.
- [25] Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirose, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M. and Tabata, S. (1996) *DNA Res.* 3, 109–136.
- [26] von Heijne, G. and Nishikawa, K. (1991) *FEBS Lett.* 278, 1–3.
- [27] Nagai, A., Suzuki, K., Ward, E., Moyer, M., Mano, J., Beck, J., Tada, S., Hashimoto, M., Chang, J.-Y., Ryals, J., Scheidegger, A. and Ohta, D. (1993) in: *Research in Photosynthesis*, pp. 95–98, Kluwer Academic, Dordrecht.
- [28] Brenner, M. and Ames, B.N. (1971) in: *Metabolic Pathways*, Vol. 5. *Metabolic Regulation*, pp. 349–387, Academic Press, New York.