Molecular Characterization of a Second Copy of Holocarboxylase Synthetase Gene (hcs2) in Arabidopsis thaliana*

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Holocarboxylase synthetase (HCS), catalyzing the covalent attachment of biotin, is ubiquitously represented in living organisms. Indeed, the biotinylation is a post-translational modification that allows the transformation of inactive biotin-dependent carboxylases, which are committed in fundamental metabolisms such as fatty acid synthesis, into their active holo form. Among other living organisms, plants present a peculiarly complex situation. In pea, HCS activity has been detected in three subcellular compartments and the systematic sequencing of the Arabidopsis genome revealed the occurrence of two hcs genes (hcs1 and hcs2). Hcs1 gene product had been previously characterized at molecular and biochemical levels. Here, by PCR amplification, we cloned an hcs2 cDNA from Arabidopsis thaliana (Ws ecotype) mRNA. We observed the occurrence of multiple cDNA forms which resulted from the alternative splicing of hcs2 mRNA. Furthermore, we evidenced a nucleotide polymorphism at the hcs2 gene within the Ws ecotype, which affected splicing of hcs2 mRNA. This contrasted sharply with the situation at hcs1 locus. However, this polymorphism had no apparent effect on total HCS activity in planta. Finally, hcs2 mRNAs were found 4-fold less abundant than hcs1 mRNA and the most abundant hcs2 mRNA spliced variant should code for a truncated protein. We discuss the possible role of such a multiplicity of putative HCS proteins in plants and discuss the involvement of each of hcs genes in the correct realization of biotinylation.

Biotin (vitamin H) is a cofactor for some carboxylases and decarboxylases (1). The biotinylation of these enzymes is a post-translational modification, which allows the transformation of inactive apo-proteins into their active holo forms. The covalent attachment of biotin is catalyzed by biotin protein ligase also called holocarboxylase synthetase (HCS). 1 D-biotin is incorporated to a specific Lys residue of newly synthesized apo enzyme, via an amide linkage between the biotin carboxyl group and a unique ε-amino group of Lys residue (2). It occurs in two steps, Equations 1 and 2 as follows,

\[ \text{d-Biotin} + \text{ATP} \rightarrow \text{d-biotinyl-5'-AMP} + \text{PP}, \]

(Eq. 1)

\[ \text{d-Biotinyl-5'-AMP} + \text{acarboxylase} \rightarrow \text{holocarboxylase} + \text{AMP} \]  

In Escherichia coli, there is only one biotin-dependent carboxylase, the acetyl-CoA carboxylase. It is biotylated by HCS also called BirA. The BirA gene has been cloned and the protein purified and extensively studied (for a review, see Ref. 3). This is a model system for the biochemical study of biotinylation. However, if the ligases from other bacterial sources are very similar to the E. coli enzyme, their eukaryotic counterparts appear quite different. On the one hand, there is more than only one biotin-dependent carboxylase in eukaryotic cells, and hence, there are different substrates for the biotinylation reaction. This raises the issue of substrate specificity of this reaction. On the other hand, the cytoplasm of eukaryotic cells is compartmentalized and the occurrence of biotin-dependent carboxylases in different subcellular compartments complicates the understanding of their biotinylation. In mammalian cells, HCS activity is located in both cytosol and mitochondria (4). In human cells, there are four carboxylases requiring biotin as a prosthetic group (acetyl-CoA carboxylase in the cytosol and pyruvate carboxylase, 3-methylcrotonyl-CoA carboxylase, and propionyl-CoA carboxylase in mitochondria). Only one gene coding for HCS had been evidenced (5, 6). As a consequence, in humans, mitochondrial and cytosolic HCS activities should result from alternative splicing of the corresponding mRNA. Furthermore, different HCS polypeptides had been detected in cytosol that could result from the alternative use of three translation initiation codons (7). In yeast, only one hcs gene has been isolated and code for the cytosolic activity of HCS, enabling the biotinylation of both carboxylases detected in yeast cytosol (acetyl-CoA carboxylase and pyruvate carboxylase) (8).

In the plant kingdom, HCS activity has been detected in three subcellular compartments (cytosol, mitochondrion, and plastid) from pea leaves (9). This is therefore the most complex situation described. These locations are in agreement with the presence of carboxylases in each of the subcellular compartments (acetyl-CoA carboxylase and geranyl-CoA carboxylase in plastid, acetyl-CoA carboxylase in cytosol, and methylcrotonyl-CoA carboxylase in mitochondria) (for a review, see Ref. 10). A cDNA encoding HCS has been isolated and characterized in Arabidopsis thaliana (9). The product of the gene has been biochemically studied and localized in plastids (11). The cytosolic and mitochondrial HCS activities remain unexplained. In the present study, we intended to clone a cDNA resulting from the expression of a new hcs gene (hcs2) using RT-PCR. We observed an unexpected diversity of messenger variants resulting from the transcription of this gene. They come from hcs2 mRNA alternative splicing. We investigated the genomic organization of hcs2. We evidenced different allelic isoforms of the hcs2 gene and studied the effects of this polymorphism on hcs1 and hcs2 expression and HCS activities.

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1 The abbreviations used are: HCS, holocarboxylase synthetase; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; SNP, single nucleotide polymorphism.
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Table I

| Primer name | Primer sequence | Annealing temperature |
|-------------|-----------------|-----------------------|
| hcs1 and hcs2 cDNA cloning | 5'-CGTATGGACAGTCTGCTCGAG-3' | 55 °C |
| hcs2.met | 5'-GGTATGGACAGTCTGCTCGAG-3' | 55 °C |
| hcs2.stop | 5'-ATGGAGACAGTCTGCTCGAG-3' | 55 °C |
| hcs1.met | 5'-CAAGCGCAAGCATCTTTCACCCGG-3' | 55 °C |
| hcs1.stop | 5'-CAAGCGCAAGCATCTTTCACCCGG-3' | 55 °C |
| hcs2 E3 | 5'-GAGACAAAGAGCACTGGGTGACG-3' | 60 °C |
| hcs1 E4 | 5'-GCCTGTTACTATAGGACATGAC-3' | 60 °C |

**Real-time PCR**

- **SNP-5**: 5'-GGATGCTTACTATAGGACATGAC-3' 60 °C
- **SNP-ag-3**: 5'-GCCAAGAAGATCTACCCGG-3' 55 °C
- **SNP-gg-3**: 5'-GCCAAGAAGATCTACCCGG-3' 55 °C
- **Ac5**: 5'-GGATGCTTACTATAGGACATGAC-3' 55 °C
- **Act5**: 5'-GGATGCTTACTATAGGACATGAC-3' 55 °C
- **act2.Q.5**: 5'-GCCAAGAAGATCTACCCGG-3' 60 °C
- **hcs1.Q.5**: 5'-GCCAAGAAGATCTACCCGG-3' 60 °C
- **hcs2.Q.3**: 5'-GCCAAGAAGATCTACCCGG-3' 60 °C
- **hcs1.Q.3**: 5'-GCCAAGAAGATCTACCCGG-3' 60 °C
- **E2.AA**: 5'-CACTACAGGTGTTTCTCATAA-3' 50 °C
- **E2.TT**: 5'-CACTACAGGTGTTTCTCATAA-3' 50 °C
- **E4**: 5'-AAGACAAACCCCTTGAGATCTTCAAA-3' 50 °C
- **E5.GG**: 5'-AAAAATGTGACTACGGGCA-3' 50 °C
- **E5.CT**: 5'-AAAAATGTGACTACGGGCA-3' 50 °C
- **E10**: 5'-GGTTACTACTGCGTGATCAG-3' 50 °C

**cac1 cloning and expression**

- 5'-CAC1: 5'-GGTATGACTGCTGATCCACGTAAGTGAGTGC-3' 50 °C
- 3'-CAC1: 5'-GGTATGACTGCTGATCCACGTAAGTGAGTGC-3' 50 °C

* The annealing temperature for the real-time PCR reactions is indicated.

**Experimental Procedures**

Media, Bacterial Strains, and Chemicals—Biotin (33 Ci/mmol) was purchased from Amersham Biosciences Inc. Isopropl-1-β-D-galactopyranoside was obtained from Bioprobe Systems. All other chemicals were obtained from Sigma Chimie SARL.

Temperature-sensitive E. coli birA15 mutant (strain BM4050) was generously provided by Dr. A. M. Campbell. Mutations in the birA gene affect the biotin ligase function of the BirA protein resulting in biotin auxotrophy. The mutant had been lysogenized with the helper phage DE3 lysogenization kit from Novagen (11). The resulting E. coli birA15(DE3) harboring a copy of the T7 RNA polymerase gene, using the Temperature-sensitive E. coli birA

**Plant Material**—Arabidopsis thaliana (ecotype Wassilewskija) plants were grown in soil under greenhouse conditions (23 °C with a 16-h photoperiod, and a light intensity of 200 μmol of photons m⁻² s⁻¹) until harvested for analysis.

**Synthetic Oligonucleotides**—The oligonucleotide primers utilized in this study are listed in Table I.

**Cloning of Arabidopsis hcs2 and hcs1 cDNAs**—Poly(A) mRNAs from Arabidopsis leaves were prepared using the Stratagene's mRNA Isolation System, according to the manufacturer's instructions (Novagen). First strand cDNA was synthesized from 250 ng of mRNA with a final volume of 20 μl using Oligo(dT)₂₀ primers (Thermoscript RT-PCR System, Invitrogen). PCR reaction was then conducted using 1 unit of Pwo polymerase (Roche Molecular Biochemicals). PCRs were performed with two pairs of nested adaptator primers and specific primers in exon 3 of hcs2 (Hcs2.E3) and genomic specific primer in exon 4 of hcs1 (Hcs1.E4). The resulting PCR products were cloned into pCRScript and sequenced.

**Cloning of Arabidopsis hcs1 and hcs2 Genes**—Full-length hcs1 and hcs2 genes were amplified by PCR from Arabidopsis leaves total DNA extracted by the CTAB method (12). hcs1 amplification was done using hcs1.met and hcs1.stop primers and hcs2 amplification using hcs2.met and hcs2.stop primers. The resulting PCR products were cloned into pCRBlunt-TOPO vector (Invitrogen) and sequenced. Partial hcs2 gene fragment was amplified by PCR using hcs2.exon7 and hcs2.stop primers and used for further restriction pattern polymorphism analysis (HPaII and BstNI digestions).

**Plant Material**—Arabidopsis thaliana (ecotype Wassilewskija) plants were grown in soil under greenhouse conditions (23 °C with a 16-h photoperiod, and a light intensity of 200 μmol of photons m⁻² s⁻¹) until harvested for analysis.

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**Identification of hcs1 and hcs2 Transcription Start Site**—The transcription start site of hcs1 and hcs2 was determined by 5’-RACE using Arabidopsis leaves cDNAs (GeneRacer Kit, Invitrogen). Two sequential PCRs were performed with two pairs of nested adaptator primers and gene-specific primers in exon 3 of hcs2 (Hcs2.E3) and genomic specific primer in exon 4 of hcs1 (Hcs1.E4). The resulting PCR products were cloned into pCRScript and sequenced.
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FIG. 1. The genomic structure of the hcs1 and hcs2 genes. A, schematic diagram of hcs1 and hcs2 loci. Boxes and lines are exons and introns. The translation start site (ATG) and stop codon (TGA) are indicated. B, amino acid sequence comparison between Arabidopsis HCS1 (accession number AAC49706) and HCS2 (conceptual translation of the putative mRNA). Identical amino acids are shaded in yellow. Region I corresponds to the catalytic core domain. Boxed amino acids are implied in biotin binding (identified in BirA, E. coli) (17). Region II, shaded in green, corresponds to the PDGNSF domain conserved in all eukaryotic HCSs.

manufacturer’s instructions (Novagen). First strand cDNA was synthesized from 1 μg of mRNA in a final volume of 20 μl using Oligo(dT)20 primers (Thermo script RT-PCR System, Invitrogen). The relative amount of hcs1 and hcs2 mRNAs was determined by real-time PCR using the PCR primers hcs2.Q.5, hcs2.Q.3, hcs1.Q.5, and hcs1.Q.3 (Table I). Dilutions of linear plasmid pPCRscript-hcs1 and pPCRscript-hcs2 were used as standards for quantification. Amplification of actin cDNA (accession number U39449) with actin.3 and actin.5 primers was used as internal standard of mRNA integrity and cDNA preparation.

Hcs2 Splicing Variants Quantification—Poly(A) mRNAs from leaves of plant line Ws-0-1 and plant line Ws-0-7 of Arabidopsis were prepared. First strand cDNA was synthesized and used for real-time PCR analyses as described above. Exon2/exon3 splicing variants were discriminated using E2.AA/E4 and E2.TT/E4 primers. Exon8/exon9 splicing variants were discriminated using E8.GG/E10 and E8.CT/E10 primers (Table I). Dilutions of linear PCRScript-hcs2α and PCRScript-hcs2δ were used as standards for quantification.

Activity Measurement—Samples of Arabidopsis plants were harvested, ground in liquid nitrogen with a mortar and pestle, and homogenized in 1 volume of chilled buffer A composed of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 5 mM e-aminoacaproic acid, 1 mM benzamidine-HCl, and 1 mM phenylmethylsulfonyl fluoride. After 15 min centrifugation (18,000 × g), the supernatant was collected and used for further analysis. The protein concentration of the extract was measured using the method of Bradford (13). HCS activity in the protein extracts was measured according to the protocol described by Tissot et al. (14) using purified CAC1 as the apocarboxylase substrate.

Cloning, Expression, and Purification of Recombinant Apo-CAC1 Substrate—The pET-29a+-CAC1 plasmid encoding the Arabidopsis biotin carboxyl carrier protein was constructed by means of PCR using the pYES-R cDNA library (15). Primers used for amplification (5′-CAC1 and 3′-CAC1) were synthesized in accordance with the published cDNA sequence (Ref. 16, accession number U23155). They introduced a BamHI restriction site and a XhoI restriction site. After digestion with BamHI and XhoI, the PCR fragment was subcloned into pET29a+ vector (Novagen) digested with the same enzymes. This construct encodes a fusion protein containing the CAC1 protein devoid of the chloroplast transit peptide and a 32-residue S-Tag presequence. The E. coli birA215(DE3) strain transformed with pET29-CAC1 was grown and induced at 37 °C in Luria-Bertani medium supplemented with kanamycin (50 μg/ml). When A600 reached 0.6, 1 mM isopropyl-1-β-D-galactopyranoside was added and growth was continued at 37 °C for 3–4 h. Cells were collected by centrifugation and resuspended in buffer A. They were then disrupted by sonication for 15 min at 0 °C and lysates were centrifuged at 40,000 × g for 15 min. Supernatant was incubated with S-protein-agarose in batch processing for 1 h at 25 °C with gentle shaking. Purification of the recombinant protein by cleavage of the leader sequence was achieved with a biotinylated thrombin according to the manufacturer’s instructions (Novagen).

RESULTS

Isoforms of hcs2 mRNA—The systematic sequencing of the Arabidopsis genome revealed a second hcs gene (hcs2, accession number AAF19230) which presents strong homologies with the gene encoding the plastidial HCS (hcs1, accession number AAD31371), confirming our prediction of the occurrence of distinct HCS isoforms in the plant kingdom (9, 11). The genomic organization of hcs2 is very close to that of hcs1 (Fig. 1A). Like hcs1, the hcs2 gene is predicted to be divided in 10 exons. The putative protein resulting from the conceptual translation of the putative hcs2 mRNA shares strong similarities with HCS1 (displaying 82% amino acid identity) and possesses the conserved domains of biotin ligases (Fig. 1B). Moreover, it does not present any predictable transit peptide. It could then catalyze the cytosolic HCS activity. To obtain the messenger resulting from the transcription of the hcs2 gene, mRNAs were isolated from Arabidopsis leaves and analyzed by RT-PCR. Specific oligonucleotide primers (designed hcs2.met and hcs2.stop in the 5′ and the 3′ ends of the hcs2 gene, and chosen according to ORF prediction and homologies with hcs1 gene) were used to amplify the product of hcs2 gene. Surprisingly, we noticed the occurrence of multiple cDNAs for hcs2.
PCR-assisted cloning enabled us to isolate eight distinct cDNAs (Fig. 2A). They exhibited sequence deletions or additions compared with the predicted hcs2 mRNA and were distinct from each other in 5' and 3' ends. The central part of the cDNA sequence was variant. Six cDNAs were found to encode a family of putative HCS2 proteins designated HCS2a to HCS2f. Two of these cDNAs (hcs2 g and hcs2 h) did not present any ORF. Sequence alignment of the putative HCS2 proteins shows that they all share the central sequence (from exon 3 to exon 8) corresponding to the catalytic core, whereas their COOH-terminal and NH2-terminal are divergent. Fig. 2B shows the complex structure of the NH2-terminal from the HCS2 protein family. HCS2a and HCS2d protein sequences corresponded to the predicted one. But, HCS2b and HCS2e differed from this sequence. Indeed, in hcs2b and hcs2e cDNA, we observed a 10-nucleotide insertion at the beginning of exon 3 from the sequence of intron 2 (according to the predicted structure of hcs2 gene). This created a frameshift and resulted in the production of a shorter ORF, the first initiating Met being localized in exon 1. Finally, HCS2c presented an original sequence in the NH2-terminal. The diversity of hcs2 mRNA splice variants contrasts with the occurrence of only one hcs1 mRNA isoform previously isolated by functional complementation of E. coli mutants (9). To investigate the possible occurrence of other mRNA isoforms of hcs1, we intended to clone its corresponding cDNA by means of
PCR. mRNAs were isolated from *Arabidopsis* leaves and analyzed by RT-PCR, as described for hcs2. Specific oligonucleotide primers (designed hcs1.met and hcs1.stop in the 5’ and the 3’ ends of the *hcs1* gene) were used to amplify the product of the expression of *hcs1* gene. Interestingly, under these conditions we did not evidence any other *hcs1* mRNA form than that previously characterized.

**Identification of hcs2 Transcription Start Site**—To accurately determine the translation initiation of hcs2, we intended to clone the entire 5’ sequence of hcs2 mRNA from the transcription start. By means of a 5’-RACE experiment we cloned hcs2 5’ end (Fig. 3B). This analysis evidenced two additional exons before the first ATG but did not reveal any other upstream ATG. To complete *hcs1* and *hcs2* mRNA studies, we also determined the *hcs1* transcription start by 5’-RACE (Fig. 3A). Surprisingly, we observed an *hcs1* 5’ end different from the one previously characterized (entire *hcs1* cDNA isolated by means of functional complementation (9)). A 101-nucleotide sequence from the 5’-untranslated region was absent in the clone obtained by RACE. The borders of this deleted sequence matched with the GT/AG rules of splicing in the *hcs1* gene. As a consequence, two different 5’-untranslated regions of *hcs1* are generated by alternative splicing of the same *hcs1* pre-messenger RNA.

**Are Sequence Variations of hcs2 mRNA Generated by Alternative Splicing?**—We determined the 5’ donor and 3’ acceptor splice sites of each of the *hcs2* cDNA clones identified (listed in Table III). The sequence of the introns was obtained from the *hcs2* gene sequence (accession number AAF19230). The nucleotide sequence at the 3’ acceptor splice sites of intron 2 (position 559) and intron 2’ (position 549) demonstrated that both sites are consistent with canonical GT/AG rules. This strongly suggests that *hcs2a* and *hcs2b* mRNAs are generated by alternative splicing events. We could put forward a similar alternative splicing mechanism to explain the occurrence of *hcs2f* mRNA. The splicing of a cryptic intron (intron 2bis) inside exon 2 could explain the deletion of 197 nucleotides of the exon 2. But, the 3’ acceptor splice site (position 437) of the intron 2bis do not match the AG/GT rule. Moreover, the occurrence of both 3’ ends of *hcs* cDNA clones could be explained by an alternative use of two 3’ acceptor splice sites in intron 2 (positions 2055 and 2083 in the *hcs2* gene). However, the first one (tcgg**2057**) does not match the consensus sequence (Table III). The 3’ splice site (GT) and 5’ splice site dinucleotides (AG) are almost invariable in higher plant introns (19). As a result, we could not explain all the nucleotide variations observed, by alternative mRNA splicing mechanisms.

**Identification of Allelic Variants of hcs2 Gene**—To understand the origin of the unusual spliced mRNAs, we decided to study in more detail the sequence of the *hcs2* gene. Specific oligonucleotide primers (designed hcs2.E7 and hcs2.stop, Table I) were used to amplify from genomic DNA, a 844-nucleotide fragment of the *hcs2* gene, including the nonconsensual intron 8/exon 9 border. After cloning and sequencing, we identified two different sequences, designated hcs2gg and hcs2ag (type I 844-nucleotide fragment) and hcs2ag (type II 844-nucleotide fragment) (Fig. 4A). These two sequences provided us an explanation for the occurrence of two different 3’ ends of *hcs2* cDNA. Indeed, a SNP in intron 8 of hcs2ag created a new 3’ acceptor splice site (TCCGG sequence in hcs2gg gene became TCCAG in hcs2ag gene) 28 nucleotides upstream from the former. This modification accounts for the unusual splicing event observed in hcs2a, hcs2b, and hcs2c cDNAs. Consequently, hcs2a, hcs2b, and hcs2c mRNAs should result from the transcription of a second *hcs2* gene (hcs2ag) presenting some SNP compared with the first form (hcs2gg). To identify the relationship between hcs2ag and hcs2gg, we analyzed the distribution of these two hcs2 forms in an *Arabidopsis* population. We used the endonuclease restriction profile polymorphism created by the GG/AG SNP (Fig. 4B). We analyzed 11 different *Arabidopsis* plants. Among these 11 plants, four presented the type I digestion pattern and seven presented the type II digestion pattern. None of them presented a mixed digestion pattern. As a consequence, all the analyzed plants presented only one of the two *hcs2* gene isoforms evidenced. Systematic sequencing confirmed that there was only one *hcs2* gene copy in *Arabidopsis* genome. We could then conclude that the two different isoforms of *hcs2* gene are allelic *hcs2* variants. Moreover, 11 inbred lines (Ws-0-1 to Ws-0-11) were generated from the Ws-0 accession for further analysis by planting self-seeds from the original Ws-0. We verified that each of the resulting lines preserved the digestion pattern of the parental plant. Besides, we measured the relative distribution of these alleles by real-time quantitative PCR. In a preparation of genomic DNA from a population of *Arabidopsis* plants, we quantified 45% of hcs2ag allele (data not shown). This ratio confirmed the nearly equal distribution of hcs2ag and hcs2gg in the *Arabidopsis* seed stock analyzed.

To evidence the effect of *hcs2* gene polymorphism on *hcs2* mRNA splicing, we studied the relative distribution of exon 9 splicing variants resulting from the use of two 3’ splicing acceptors (splicing type I and II, Fig. 5A). Using the real-time RT-PCR approach, we quantified each of them (Fig. 5B). In a preparation of RNAs from whole *Arabidopsis* plants group from our seed stock, we detected both splicing types but type II was slightly in the majority representing ~70% of *hcs2* mRNA. We analyzed the relative amount of each of these transcripts in different organs of *Arabidopsis* plants, with similar results (data not shown). In plant Ws-0-1 (hcs2gg allele), type II

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Nucleotides are numbered beginning at the start of each indicated exon. cDNAs possessing the same nucleotide as putative cDNA sequence are marked by dots at that site. Nucleotide changes are indicated by the appropriate letter and nucleotide deletion by a “d.” For partial cDNA sequences, absence of data is indicated by a “n.s.” (not sequenced). The putative cDNA sequence used as reference is reported according to the systematic sequencing of *Arabidopsis* genome (18).

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**Table II**

**Nucleotide polymorphism at hcs2 cDNA**

Nucleotides are numbered beginning at the start of each indicated exon. cDNAs possessing the same nucleotide as putative cDNA sequence are marked by dots at that site. Nucleotide changes are indicated by the appropriate letter and nucleotide deletion by a “d.” For partial cDNA sequences, absence of data is indicated by a “n.s.” (not sequenced). The putative cDNA sequence used as reference is reported according to the systematic sequencing of *Arabidopsis* genome (18).

| cDNA | Exon 1 | Exon 2 | Exon 4 | Exon 5 | Exon 6 | Exon 7 | Exon 8 | Exon 9 |
|------|--------|--------|--------|--------|--------|--------|--------|--------|
| hcs2a | G     | G      | T      | A      | G      | T      | A      | C      |
| hcs2b | C     | A      | A      | C      | C      | T      | T      | A      |
| hcs2c | C     | A      | C      | C      | T      | T      | T      | A      |
| hcs2d | C     | A      | C      | C      | T      | T      | A      | A      |
| hcs2e | C     | A      | C      | C      | T      | A      | A      | A      |
| hcs2f | d     | d      | d      | d      | n.s   | n.s   | n.s   | n.s   |
| hcs2g | C     | C      | n.s   | n.s   | n.s   | n.s   | n.s   | n.s   |
| hcs2h | C     | C      | n.s   | n.s   | n.s   | n.s   | n.s   | n.s   |

*n* A, indicates the occurrence of a termination signal.
amount reached 90% of total \textit{hcs2} mRNA, whereas splicing type I level did not exceed the background noise of PCR. Thus, in \textit{hcs2gg} plants, there was only one detectable splicing event that created a premature termination signal. On the contrary, in \textit{hcs2ag} allele, splicing type I was significantly detected. Its relative amount exceeded the level of splicing type II and raised 60% of total \textit{hcs2} mRNA. As a result, in \textit{hcs2ag} plants, the intron 8 SNP (nucleotide 2055) created a new splice site which entitled the detection of splicing type I of exon 9 in \textit{hcs2} mRNA. The second splice site (the only one used in \textit{hcs2gg} plants) kept on being used and explained the detection of splicing type II of exon 9 in \textit{hcs2} mRNA. Consequently, in these plants and contrary to \textit{hcs2gg} plants, alternative splicing of exon 9 created two different mRNA 3' ends and hence, two different putative NH$_2$-terminal HCS2 proteins. In summary, a nucleotide polymorphism at the \textit{hcs2} gene created an allele-dependent splicing of exon 9. To quantify this polymorphism, we sequenced the entire \textit{hcs2} gene.

\begin{table}[h]
\centering
\caption{Exon-intron junction sequence of the Arabidopsis \textit{hcs2} gene}
\begin{tabular}{|c|c|c|c|c|}
\hline
Exon & 5' Donor splice site & Intron & 3' Acceptor splice site & Exon & Occurrence in cDNA \\
\hline
1 & AG$^{90}$/gtage & 1 & ttgA$^{90}$/CC & 2 & All \\
2 & CA$^{77}$/gtacg & 2' & ttgA$^{77}$/TT & 3 & \textit{hcs2b, 2e} \\
2 & CA$^{77}$/gtacg & 2 & ttcag$^{549}$/TT & 3 & \textit{hcs2a, 2c, 2d, 2f, 2g, 2h} \\
2a & TG$^{290}$/gtaat & 2bis & ttg$^{290}$/TC & 3 & \textit{hcs2c, 2h} \\
3 & AG$^{257}$/gtag & 3 & ttcg$^{477}$/GC & 4 & All \\
4 & AG$^{657}$/gtage & 4 & tttg$^{657}$/GG & 5 & All \\
5 & TG$^{1058}$/gtata & 5 & ttcg$^{1152}$/GT & 6 & All \\
6 & AG$^{1308}$/gtag & 6 & ttcg$^{1463}$/GT & 7 & All \\
7 & AG$^{1597}$/gtag & 7 & cg$^{1607}$/GT & 8 & All \\
8 & AG$^{1974}$/gtag & 8 & gctc$^{2055}$/GG & 9 & \textit{hcs2d, 2f, 2e, 2g} \\
9 & AG$^{2134}$/gtag & 9 & ttcg$^{2055}$/GG & 9 & \textit{hcs2a, 2b, 2c, 2g, 2h} \\
\hline
\end{tabular}
\end{table}

\section*{Polymorphism at \textit{hcs2} Gene—A total of 2322 nucleotides of the \textit{hcs2} gene were sequenced for five inbred lines of \textit{A. thaliana} Ws ecotype (Ws-0-1, Ws-0-4, Ws-0-5, Ws-0-6, and Ws-0-10). This encompassed the 2251 nucleotides containing the HCS2 protein coding region plus nine introns and a portion of the flanking DNA including 23 nucleotides at the 5' end and 48 nucleotides at the 3' end of the ORF. We confirmed the results obtained by restriction patterns and obtained three different \textit{hcs2} sequences (Ws-0-4/Ws-0-6/Ws-0-10, Ws-0-1, and Ws-0-5). Furthermore, within the entire sequenced region, 21 polymorphic sites were observed. The position of all nucleotide changes found among the Ws-0-4 and Ws-0-5 \textit{hcs2} genes (\textit{hcs2ag} allele)...
of the 21 SNP observed, 11 were present in intron sequence and 10 were observed in coding sequence. Of these, five resulted in silent substitutions, three involved conservative amino acids changes, one involved nonconservative amino acid change (substitution of Ile with Asn), and one resulted in a change from a Trp to a stop codon (position 437 (exon 2)). Two SNP resulted in the creation of splice sites (position 437 (exon 2) and 2055 (intron 8)). These observations elucidated the unexplained splicing of exon 2 (in hcs2c) and unexplained splicing of exon 9 (hcs2a, hcs2b, and hcs2c). The Ws-0-1 hcs2 gene did not present any polymorphism compared with the Col-0 hcs2 gene sequence. The polymorphisms observed in the entire hcs2 gene sequence are consistent with the previously observed polymorphisms in hcs2 cDNA sequences. Indeed, the hcs2 gene from Ws-0-4 type plants presented the same exonic SNP that those previously observed in hcs2a, hcs2b, and hcs2c cDNA clones (see Table II).

To determine whether hcs2 gene polymorphism only reflected non-selective genomic variations inside the Ws ecotype or if it was hcs2 gene-specific, we investigated hcs1 gene polymorphism. A total of 2385 nucleotides of the hcs1 gene were sequenced in Ws-0-1 and Ws-0-6 plants. Ws-0-1 hcs1 gene sequence was identical to the Col-0 hcs1 gene sequence. Within the entire sequenced region, only two polymorphic sites were observed in Ws-0-6 hcs2 gene. Only one was observed in coding sequence (position 83 in exon 1), and resulted in a conservative amino acid change (substitution of His with Arg). As a result, 0.084% of nucleotides are polymorphic within hcs1 and 0.04% of the observed polymorphisms result in a change in amino acid composition. By way of comparison, 0.9% of nucleotides are polymorphic within hcs2 and 0.17% of the observed polymorphisms result in a change in amino acid composition.

We measured HCS activity in Ws-0-1 to Ws-0-11 plant lines. Although hcs2 gene sequence polymorphism have severe con-

**FIG. 4.** Analysis of the 3' terminal of the hcs2 gene. A, schematic representation of the two 3' sequences of the hcs2 gene and their corresponding spliced mRNA. A 844-nucleotide PCR fragment (from the beginning of intron 7 to the termination signal) was cloned and sequenced. Two different sequences were evidenced: type I sequence (hcs2gg allele) and type II sequence (hcs2ga allele). The cDNA clones matching the putative mRNA sequence are indicated at the beginning of the sequence. Lines and boxes in the hcs2 gene represent exons and introns, respectively. Arrowheads indicate sites of polymorphism. B, schematic representation of type I and type II fragment digestion profiles. Genomic DNA was extracted from 11 individual Arabidopsis plants. For each of these plants, a 844-nucleotide PCR fragment was tested for BstNI and HpaII digestion. The size of the theoretical digested fragment is indicated. The digestion patterns corresponding to the Ws-0-3 plant and Ws-0-5 plant are presented. For each of them, other plants presenting the same profile are indicated.

**FIG. 5.** Real-time RT-PCR quantification of spliced variants of exon 9. A, a schematic representation of E8.GG, E8.CT, and E10 oligonucleotides positions on exon 8/exon 9 junction and on exon 10. E8.GG oligonucleotide enabled the quantification of type I splicing event. E8.CT primer oligonucleotide enabled the quantification of type II splicing event. B, real-time RT-PCR experiment on poly(A)+ RNA from Arabidopsis aerial parts from a mixture of plants from Ws-0-1 and Ws-0-6 plants using splicing type-specific primers. Data (percentage of the total amplification) are means of three independent experiments performed with three cDNA dilutions ± S.D. β-Actin has been used as internal standard of RNA integrity and quantity.

is listed in Table IV. Ws-0-6 and Ws-0-10 hcs2 genes presented the same 20 polymorphic sites than those reported in Ws-0-4. Of the 21 SNP observed, 11 were present in intron sequence and 10 were observed in coding sequence. Of these, five resulted in silent substitutions, three involved conservative amino acids changes, one involved nonconservative amino acid change (substitution of Ile with Asn), and one resulted in a change from a Trp to a stop codon (position 437 (exon 2)). Two SNP resulted in the creation of splice sites (position 437 (exon 2) and 2055 (intron 8)). These observations elucidated the unexplained splicing of exon 2 (in hcs2c) and unexplained splicing of exon 9 (hcs2a, hcs2b, and hcs2c). The Ws-0-1 hcs2 gene did not present any polymorphism compared with the Col-0 hcs2 gene sequence. The polymorphisms observed in the entire hcs2 gene sequence are consistent with the previously observed polymorphisms in hcs2 cDNA sequences. Indeed, the hcs2 gene from Ws-0-4 type plants presented the same exonic SNP that those previously observed in hcs2a, hcs2b, and hcs2c cDNA clones (see Table II).

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Table IV
Nucleotide polymorphism at hcs2 gene

| Ecotype | 104 | 114 | 158 | 8 | 665 | 8 | 303 | 8 | 951 | 8 | 1051 | 8 | 1145 | 15 | 1270 | 8 | 1378 | 6 | 1452 | 8 | 1480 | 8 | 1915 | 8 | 2050 | 6 |
|---------|-----|-----|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|
|         |       |      |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |
| Col-0   | T    | G    | G    | A | A    | G | T    | C | G    | G | A    | A | G    | G | G    | G | G    | G | G    | G | G    | G | G    |
| Ws-0—4 | A    | T    | C    | A | C    | C | T    | T | A    | T | A    | A | T    | A | G    | G | C    | A | G    | A | A    | A | A    |
| Ws-0—5 | A    | T    | C    | A | C    | C | T    | T | A    | A | A    | A | C    | A | G    | G | C    | A | G    | A | A    | A | A    |

* Nucleotides marked indicate sites at which splice site changes occur.

| Sequences on hcs2 mRNA splicing and COOH-terminal HCS2 protein sequence, the in vitro assay performed with CAC1 as acceptor substrate did not evidence any significant variation of total HCS activity correlated with the nature of hcs2 allele (data not shown). Moreover, plants had no discernible phenotype, whatever hcs2 allele they bore.

Expression of hcs Genes—To have insight into the physiological function of each hcs gene, we intended to study their expression patterns in various plant organs. By means of quantitative RT-PCR measurements, we evaluated hcs1 and hcs2 transcripts abundance (Fig. 6A). Hcs1 and hcs2 mRNAs were detected in all analyzed organs (roots (R), stems (St), leaves (L), flowers (F), siliques (Si), and seeds (S)) using hcs1- and hcs2-specific primers (A). Data are means of three independent experiments performed with four cDNA dilutions ± S.D. —Actin has been used as internal standard of RNA integrity and cDNA preparation. HCS activity measurement has been performed on protein extracted from the same organs (B).

To determine more accurately the hcs2 expression profile, we studied the distribution of the two alternative splice variants of intron 2/exon 3 border (Fig. 7). Two different 3′ acceptor splice sites distant from 10 nucleotides are alternatively used. We quantified each of the resultant messengers by way of real-time PCR. Surprisingly, type III was the most abundant splicing type, resulting in the creation of a 10-nucleotide longer exon 3 (exon 3′) and in a frameshift (see Fig. 2). The putative proteins associated with this splicing are NH2-terminal truncated...
This splicing distribution did not vary with the tissue experienced (Fig. 7B) and was not dependent on the hcs2 gene allele (Fig. 7C).

**DISCUSSION**

When making an inventory of the parties involved in betaine metabolism, we observed a peculiarly complex situation in plants. HCS activity had been detected in three subcellular compartments (9) and the systematic sequencing of *Arabidopsis* genome revealed the occurrence of two hcs genes (hcs1 and hcs2) (18). Hcs1 was previously characterized and found to encode plastidial isoform (9, 11). Here, we focused our attention on the hcs2 gene and its products. The salient facts of our investigation are (i) hcs2 gene is expressed and subjected to alternative splicing; (ii) hcs2 gene is characterized by high level of nucleotide polymorphism inside Ws ecotype; (iii) the level of polymorphism at hcs2 locus contrasts sharply with that observed at hcs1 locus; (iv) the observed polymorphism affects splicing of hcs2 mRNA but does not have any effect on HCS activity level in whole plant extracts nor on plant phenotype; (v) total hcs2 mRNA level is 4-fold lesser than hcs1 mRNA; and (vi) the most abundant hcs2 mRNA spliced variant should encode a truncated protein.

**Are HCS2 Putative Proteins New Forms of Eukaryotic HCS?**—We evidenced different isoforms of hcs2 mRNA which could encode putative proteins variable in amino- and carboxyterminal. We were not able to test the occurrence of these proteins in a cellular extract of *Arabidopsis* although we had at our disposal the HCS1 antibody. This antibody cross-reacted with HCS2 recombinant proteins (data not shown) but the HCS proteins concentration in plant extracts was too low to be accurately detected. These putative proteins can be classified according to their domains organization (20) (Fig. 8A). Whenever hcs2 allele, the most represented putative HCS2 protein (according to its corresponding mRNA relative abundance (hcs2b for hcs2ag and hcs2e for hcs2gg)) should be NH2-terminal truncated. It is an original eukaryotic HCS organization and this NH2-terminal structure is related to some bacterial ligases which do not present the helix-turn-helix domain of BirA (involved in DNA fixation and biotin operon regulation) and present no more than the catalytic core domain in NH2-terminal. We evidenced two different putative COOH-terminal HCS2 protein sequences. One presents the PGDNSF eukaryotic specific domain (observed in HCS2a, HCS2b and HCS2c) while the other is devoid of this conserved sequence. This partition is allele-dependent, as far as hcs2gg allele plants could not produce the COOH-terminal complete HCS2 protein. The function of this domain is not determined nor its exact involvement in HCS activity, but human HCS deleted of its last 31 amino acids (comprising the PDGNSF sequence) lost all activity (21). However, for the *Arabidopsis* enzyme, which is closer to its bacterial counterpart, the HCS2 proteins depleted from this sequence (HCS2d, -e, and -f) could remain active. The significance of several splice variants of hcs2 is unknown. As this alternative splicing affects the 5’ of hcs2 mRNA and should modify the NH2 terminus of HCS2 protein, it could produce proteins with different subcellular distributions (Fig. 8). Indeed, prediction programs such as Psort, chloroP, and mitoP give putative HCS2a, HCS2e, and HCS2d a cytosolic localization and HCS2f a mitochondrial localization. These supposed localizations are in good agreement with HCS activities found in plant cell cytosol and mitochondria (9) and matches well with sites of accumulation of some biotin-containing proteins in plant cells (10). According to the prediction programs, HCS2b and HCS2e should present a vesicular path-
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way localization (Fig. 8). It is interesting to notice that in Arabidopsis, two “cytosolic” ACCase isoforms have been described (22). The corresponding gene sequences are very close, but one of them (accession number AAG40564) should present a NH2-terminal extension which is predicted to be membraneous. Moreover, prediction programs (targetP, chloroP) give this protein a reticular localization. Thus, the HCS2 NH2-terminal truncated isoform could be its specific ligase.

Alternatively, the active domain of the hcs2 gene could come down to the sequence between exon 3 and exon 9. So, exon 2 and exon 10, subjected to deleterious alternative splicing could be useless in HCS2 protein. This would give the HCS2 protein peculiar properties (substrate specificity and kinetic properties) different from HCS1. It could influence substrate specificity of each of them, in particular with respect to biotinylatation sites different from the consensus one. Indeed, a cytosolic seed-specific biotinylated protein (SBP65) with no carboxylase activity had been evidenced in pea (23). This protein presents an atypical biotinylatation site. SBP65 homologue has been found in Arabidopsis seeds (24). It is also possible that other original biotinylated proteins exist in Arabidopsis that could explain the diversity of HCS isoforms in plant cells.

Hcs2 Gene Presents a Nucleotide Polymorphism—When comparing Col-0 and Wa-0 hcs2 sequence, 0.9% of nucleotides are different and 0.17% of the observed polymorphisms result in a change in amino acid composition. It is a significant level of intraspecific sequence polymorphism. By way of comparison, such level of polymorphism was found within the RPS2 gene (1.26%) (25) or at the RPP5 disease resistance loci (26). These are examples of sequence polymorphism affecting plant Resistance (R) genes which are currently subjected to such genetic variation (27). Bergelson et al. (28) examined levels of polymorphism for three nuclear genes within and among the population of Arabidopsis. For these nuclear genes, the average nucleotide diversity was 0.04% within the Arabidopsis population and 0.14% between populations. The polymorphism observed in the hcs2 gene could have deleterious effects on HCS protein integrity because of its effect on hcs2 mRNA splicing. Despite these severe consequences on hcs2 mRNA and HCS2 protein sequences, the ratio of each hcs2 alleles was found to be around 50% in the Ws population we had at our disposal, and all the plants analyzed were homozygous for this trait. Moreover, there were no apparent consequences of this allelic diversity on ACCase activity and plant phenotypes, which probably indicate relatively weak functional constraint on the hcs2 gene. A possibility is that protein HCS2 function is redundant with that of HCS1. These two genes have very close sequence homology. Thus, one cannot exclude that hcs2 could be an inactive pseudogene in Arabidopsis. It would be the relic of a former duplication event of the hcs gene consistent with the general duplication of Arabidopsis genome (18) leaving, after evolution, only one resulting copy (hcs1) active. This possibility would be consistent with the observation that some of the spliced HCS2 isoforms are allele-dependent (HCS2a, -c, -d, -f; Fig. 8B). We have isolated by RACE experiment, an hcs1 mRNA 5′ end differing from the formerly studied. The observed deletion could influence the ATG choice. Indeed, two in-frame ATG could be used as initiation codons (9) in the hcs1 mRNA (see also Fig. 2A). In the present case, the observed deletion covered a zone of repeated sequence that can create some secondary structure. The structure of the 5′-untranslated region can influence the initiation of translation (29). The first ATG gives HCS1 a plastidial localization (11), the second could give HCS1 a cytosolic localization. As a result, hcs1 gene could be sufficient to explain cytosolic and plastidial HCS activities. This situation had been observed in human, where only one hcs gene and two subcellular localizations of HCS activity (mitochondrial and cytosolic) were evidenced (6). Another possibility is that HCS2 is useful on occasion, but such occasions may not occur during the lifetime of the average plant. For example, Arabidopsis adh is a nonessential gene that is thought to be subjected to balancing selection for two haplotypes (30). Likewise, Henikoff and Comai (31) observed an allelic diversity of CMT1, some of the alleles presenting deleterious mutations. They put forward such an hypothesis to explain the maintenance of both alleles. In our case, hcs2 could encode an isoform implied, for example, in specific activation of cytosolic ACCase, committed in flavonoids or long chain fatty acid synthesis, restricted in the epidermal cells. As a result, HCS2 deficiency could have any effect apart from the occurrence of an environmental stress such as UV exposition or pathogen attack.

Studies of the biochemical and kinetic properties of HCS2 recombinant proteins and of their subcellular localization in the plant cell, which are currently in progress in our laboratory, will entitle us to have insight into their physiological function. Moreover, reverse genetic studies and particularly characterization of knockout hcs1 and hcs2 mutants, which have been initiated, will give us some elements to unravel the role of each of Arabidopsis hcs gene in the realization of protein biotinylation.

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